

A STUDY OF THE FUSION
OF HIGHER PLANT PROTOPLASTS.

by

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Abbreviations and Conventions

cm	centimetre(s)
g	gramme(s)
xg	centrifugal force
h	hour(s)
HAU	Haemagglutination units
kV	kilovolts
M	molarity
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mg	milligramme(s)
μ	micron(s)
μ g	microgramme(s)
nm	nanometre(s)
ppm	parts per million
rpm	revolutions per minute
sec	second(s)
IAA	indole acetic acid
1-NAA	1-napthalene acetic acid
24-D	2,4-Dichlorophenoxy acetic acid
6-BAP	6-benzyl amino purine

(Any other abbreviations are qualified in the text).

Solutions

Concentrations of solutions are defined by molarity (M) or by percentage expressed as weight in g, or volume in ml, per 100 ml of solvent. (w/v, v/v respectively). Unless otherwise stated solutions are aqueous. Concentrations are not redefined if recently mentioned.

References

Papers are initially referred to by the names of all the authors, subsequently by the first author, et al.

ABSTRACTA STUDY OF THE FUSION OF HIGHER PLANT PROTOPLASTSLyndsey A. Withers

Protoplasts and spontaneous fusion bodies can be isolated from a number of tissues, by treating the tissues with a mixture of macerating and cellulolytic enzymes. The shape of the spontaneous fusion bodies frequently reflects the parental tissue structure. An electron microscopic study of the treatment of the oat root tip and tobacco leaf with enzymes, reveals that spontaneous fusion is brought about by the expansion of plasmodesmatal connexions within the tissue. Specific treatments before and during enzyme treatment can affect the level of spontaneous fusion.

The culture of spontaneous fusion bodies in solid media is less successful than in liquid media, where cytoplasmic reorganization, wall regeneration and division occur. The pattern of division is irregular and may not be mitotic. The level of multinucleation in cultures declines with time. Whilst some spontaneous fusion bodies decline and others may subdivide, microdensitometric evidence suggests that nuclear fusion or close aggregation may be occurring. There is, however, no microscopic evidence for nuclear fusion.

The fusion of originally separate, uninucleate protoplasts can be induced by treatment with sodium nitrate. Membrane adhesion and fusion are followed by organelle redistribution and vacuolar fusion. No interspecific fusion bodies are formed and the intraspecific fusion products demonstrate a low viability. Other salts induce protoplast adhesion and abnormal plasmalemmar activity but not fusion. Similarly, Concanavalin A and lysozyme induce strong adhesion but no fusion.

Treatment of protoplasts with Sendai virus can induce adhesion and eventual lysis, with membrane fusion as a likely intervening stage. Lysolecithin induces a similar reaction. It is possible that such reactions could be controlled to successfully induce protoplast fusion.

CHAPTER ONE

INTRODUCTION

One of the most distinctive characteristics of the higher plant is its structural rigidity, which is maintained by the properties of the two continua within the plant. The cell wall continuum lends a base level of support which is augmented, in primary tissues, by the turgor of the cytoplasmic continuum, the symplasm.

The relationships of the two continua are such that (in somatic tissues) the cytoplasm is compartmentalized into uninucleate units, protoplasts. The continuity of the symplasm is maintained solely by small plasmodesmatal connexions traversing the cell walls, linking adjacent protoplasts. The protoplast units have an organellar content dependent upon the location and degree of differentiation of the protoplast/cell.

The limitations imposed by such a structure are only too apparent to the cell biologist and biochemist. The development of plant tissue culture techniques has facilitated the study of structural units other than the whole plant or organ, but the inflexibility of the cellular pattern is still present. The existence of the protoplast within the cell wall and its ability to detach from the wall under plasmolysing conditions have been recognised for almost a century. (Hanstein, 1880). The combination of plasmolysis and mechanical disruption of the tissues permitted the release of a limited number of protoplasts (Klerker, 1892) which could be studied 'in vitro'. The work was, however, greatly hindered by paucity of material.

The development of enzymatic methods for isolating protoplasts, (Cooking, 1960) revolutionised techniques. The units of the symplasm could, on a large scale, be isolated, freed from the structural and functional constraints imposed by the cell walls. Isolated protoplasts

have lent themselves to a very wide range of studies, dealing with aspects of enzyme biochemistry, (Jones, Armstrong and Taiz, 1973), 'de novo' cell wall synthesis, (Grout, 1973), virus uptake, (Takebe and Otsuki, 1969, Cocking, 1970 (review)), membrane properties, (Ruesink, 1973) culture and morphogenesis, (Takebe, Labib, Melchers, 1971) and the most important in the present context, fusion and somatic hybridization. (Power, Cummins and Cocking, 1970; Carlson, 1973).

The hybridization of plants has, in the past, been executed using the normal processes of sexual reproduction, which, although wide in application, are limited to crosses between genetically compatible species. Somatic hybridization, by the interspecific fusion of isolated protoplasts has been suggested as a means of overcoming this limitation. A high level of sophistication has been achieved in the techniques of animal cell hybridization, (Harris, 1970), and it was hoped to follow a somewhat parallel course using plant protoplasts. The added advantage of plant cell totipotency, (Steward, Ammirato and Mapes, 1970), lends feasibility to the scheme, the regeneration of a whole plant from the somatic hybrid protoplast being theoretically possible.

More than half a century ago Kuster (1909) described the fusion of plasmolytically produced subprotoplasts within the cell, and later (Kuster, 1910), was able to fuse isolated protoplasts - by mechanical manipulation. More recently, Power et al (1970), induced the fusion of isolated protoplasts by treating them with sodium nitrate. These workers noted that some fusion was occurring spontaneously, multinucleate units being liberated during the enzyme degradation of the original tissue. Few clues to the processes involved in the induced and spontaneous fusion

could be found in a light microscopic study. An electron microscopic study was clearly necessitated.

The induced fusion of protoplasts by sodium nitrate treatment has become a widely used technique. It has been attempted, however, in this study, to extend the range of fusion inducing techniques using salts other than sodium nitrate, a number of organic compounds, some of which will induce animal cell fusion, and Sendai virus.

Whole plants have been successfully regenerated from single, uninucleate protoplasts. (Takebe, et al, 1971) and apparently from an induced interspecific hybrid. (Carlson, 1973). The intermediate stages of multinucleation and the mixing of cytoplasms of different origin must be critical stages in the regeneration process. Clues to the possible behaviour of multinucleate protoplasts can be drawn from the studies of animal cell hybridization and the naturally occurring syncytia in higher plants, fungi and slime moulds and protistans. As a contribution towards an understanding of the nuclear and cytoplasmic interactions involved in protoplast hybridization, a study of the behaviour in culture of intraspecific multinucleate protoplasts has been carried out.

The work presented in this thesis is initially dealt with in two sections: Materials and methods, and results relating to spontaneous fusion, being followed by those relating to induced fusion. A common discussion is presented.

To aid reading, section sheets (gold) precede each chapter detailing the contents, and summaries are included at intervals, (buff sheets).

CHAPTER TWO

THE SPONTANEOUS FUSION OF PROTOPLASTS:

MATERIALS and METHODS

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(Suppliers of non-standard chemicals are named in text)

2.1 GROWTH OF PLANT MATERIAL

2.1.1 Oat Seedling

Seeds of Avena sativa cv Victory were surface sterilized using one of the following techniques:

- a. Dehusked seeds were imbibed in running tap water for 6h, thoroughly dried with tissues and placed in pre-cooled 50% v/v sulphuric acid. After 30 min at -15°C (in deep-freeze) the seeds were washed four times with copious sterile distilled water.
- b. Dehusked seeds were imbibed overnight in running tap water, drained and soaked in 1% v/v Cetavlon (I.C.I. Ltd.) for 1 min. The seeds were transferred to 0.1% w/v aqueous mercuric chloride for 20 min then washed as above.
- c. Dehusked seeds were soaked for 15 min in 0.2% w/v mercurous chloride, then imbibed overnight in running tap water. The seeds were resterilized for 3 min with the mercurous chloride then washed as above.
- d. Dehusked seeds were imbibed overnight in running tap water, rinsed for 30 sec in 70% v/v ethanol, then surface sterilised for 30 min in 5% v/v calcium hypochlorite (10-14% available chlorine). The seeds were then washed as above. (Sources: J. B. Power, 1971, Ph.D. Thesis, University of Nottingham: A. G. Keats, personal communication).

Each of the methods was tried and compared for survival, sterility and degree of tissue deformation. Method (a) was finally selected giving minimum deformation, high sterility with an acceptable survival rate (70%).

The surface sterilized seeds were germinated in sterile petri

dishes on damp filter paper, for 3 days at 20°C in the dark. After this time primary roots of up to 3 cm in length were produced.

2.1.2 Maize seedling

Seeds of Zea mays cv Kelvedon glory were surface sterilized according to Method 2.1.1a and germinated under conditions described above but for a period of up to 6 days by which time the coleoptile was well developed.

2.1.3 Onion

Bulbs of Allium cepa cv Stuttgarter giant, of approximately 10 cm in diameter, were grown on wire netting suspended over a regularly renewed water bath and under fluorescent lighting at 1500 lux. Root induction occurred at approximately 10 days followed by leaf growth. The material for experimentation was selected by growth stage rather than age since there was little uniformity in rate of growth.

2.1.4 Tobacco

Plants of Nicotiana tabacum cv Xanthi NC were grown in the Botanic Garden greenhouses at Nottingham University. Seeds were germinated in Levington all-peat compost, transferred to fresh compost at 21 days and then at 42 days to a final potting medium of Alexpeat compost. Initial growth was under fluorescent lighting of 10,000 lux at leaf surface and from approximately 45 days the plants were transferred to illumination under Complex plant irradiators using Cryselcor 400 watt MBF/U lamps again at 10,000 lux. All growth was at 25°C-28°C at a day length of 16h. Plants were selected at required stages of growth.

2.2 PROTOPLAST ISOLATION

A general protocol for protoplast isolation from plant tissues

involves harvesting plant material, carrying out necessary pretreatments such as surface sterilization and tissue dissection, enzyme incubation and finally protoplast harvesting and washing. Details of the various isolation procedures used are given below (2.2.2 - 2.2.5.)

2.2.1 Enzymes

Two classes of enzymes were used, all commercially manufactured and used in their crude form with the exception of purified Meicelase. The crude enzymes are in fact complex enzyme systems, their use and nomenclature deriving from their predominant rather than entire properties.

Macerozyme (Kinki Yakult Manuf. Co. Ltd., Nishinomiya, Japan.)

This enzyme, isolated from the fungus Rhizopium spp. contains mainly a pectinase, the other enzymes present including ribonuclease. (R.H.A. Coutts, 1973, Ph.D. Thesis, University of Nottingham). The pectinase has a pH optimum of 4-6.5 and a temperature optimum of 40°C. (J. B. Power, 1971, Ph.D. Thesis, University of Nottingham).

Onozuka P1500 and 3000 (Kinki Yakult Manuf. Co. Ltd.) These form part of a series of enzymes of increasing cellulolytic activity, all isolated from the fungus Trichoderma viride. The suffix code number refers to activity as assayed by filter paper degradation under optimal conditions. The series covers 500 to 5000 units of activity. They contain, in addition to several cellulases, appreciable amounts of hemicellulase plus glucosidase, xylanase, glucanase, mannanase, galacturonase and macerating enzymes. The cellulase optima are at pH 4-5 and 40-50°C. (Tomiya, Suzuki and Nisizawa 1968).

Meicelase P (Meiji Seika Kaisha Ltd., Tokyo, Japan.) This extract again isolated from T. viride contains a series of cellulases, cellobiase, xylanase, amylase, glucanase, pectinase, saccharase, protease, ribonuclease, lipase and phospholipase. The optima are at pH 4-5 (according to substrate) and 45-50°C. (Meicelase (Cellulolytic Enzyme) Analytical Specifications and Test Methods, Meiji Seika Kaisha Ltd., No. 8.)

Purified Meicelase. The cellulolytic activity of Meicelase can be increased approximately tenfold by a one step purification process. (Evans and Cocking, 1973; modified from Kao, Gamborg, Miller and Keller, 1971).

Crude enzyme (15g) was dissolved overnight in 30 ml of water at 4°C. The sediment was spun down, the supernatant being loaded into a Biogel P6 column. To the column was added 5 ml of water followed by 5 ml of 3.5M sodium chloride and then the enzyme was eluted with water. The enzyme containing fractions were bulked and freeze dried. This desalting process, whilst increasing the cellulase activity actively reduces ribonuclease activity.

An enzyme incubation mixture generally consists of a mixture of enzymes dissolved by magnetic stirring in a sugar solution, the latter being necessary for protoplast stability. Other additives where included are specified. Enzyme pH optima tend to be rather low and the temperature optima high compared with the optimal conditions for tissue and protoplast survival. Therefore in the preparation of enzyme incubation mixtures compromise conditions were used, adjustments being made using 1N hydrochloric acid or 1N potassium hydroxide.

2.2.2 Oat root protoplast isolation

The terminal 3 mm of the root tip was excised under aseptic conditions (see 2.3.1) and placed, either intact or cut in two sagittally, in a drop of filtered enzyme incubation medium on a sterile slide. To prevent drying during isolation, the incubation was carried out in a sterile petri dish containing damp filter paper. Incubation was carried out at 20°C for 3-36h.

Enzyme incubation medium (Power, Cummins and Cocking, 1970)

10% w/v Onozuka P1500

5% w/v Macerozyme

20% w/v sucrose

pH unadjusted (usually 5.6-6)

Following incubation the enzyme mixture was pipetted away and replaced with several washes of 20% sucrose. The subsequent treatment of the material varied, details being given in results (3.1).

2.2.3 Maize seedling protoplast isolation

Both the root and aerial regions of the maize seedling were treated, the root essentially as in 2.2.2. Under aseptic conditions the first leaf and coleoptile were dissected from the seedling, placed separately in drops of enzyme incubation medium and finely chopped under the solution. Incubation conditions were as in 2.2.2, the incubation terminating after 6h.

Enzyme incubation medium (S. N. Chakraborty, personal communication)

3% w/v Onozuka P3000

1% w/v Macerozyme

9% w/v sorbitol

pH unadjusted (usually 5.6-6)

Post incubation washing removed the enzyme mixture which was replaced by several washings of 9% sorbitol. Care was taken to concentrate the digested material to prevent loss during the washing. Usually, swirling the drop of liquid several times was sufficient.

2.2.4. Onion protoplast isolation

The root tips, 3 mm long, were excised from roots when they had reached a length of approximately 8 cm. They were treated in a manner similar to the oat and maize root tips (2.2.2), but without the aseptic precautions.

Leaves were selected when the green region protruded approximately 30 cm from the bulb. The bulb was cut in two, the leaf being eased out after making a transverse cut to separate it from the short stem and root region. The leaf was cut into sections, the dark green bifurcated region, an intermediate light green region and a lower region, predominantly white. Cutting open the leaf pieces, the pith was scraped out to form a fourth region. This and the three outer leaf sections, finely chopped, were placed in separate 100 ml Erlenmeyer flasks containing enzyme incubation media.

Enzyme incubation media

Various media were tried, varying the cellulase component and the osmoticum;

4-6% w/v Onozuka P1500 or

3-6% w/v Meicelase P

2% w/v Macerozyme

2-20% w/v sucrose

pH 5.8

The incubation was carried out at 20°C for periods of 4-18h, some preparations being static, others on a rotary shaker at approximately 60 r.p.m.

Following incubation, the protoplasts were separated from debris by pouring through 1 μ nylon mesh (Henry Simon Ltd., Stockport, England). The protoplasts tended to separate into floating and sinking components, to be separated by careful pipetting and then transferred to fresh sucrose solution. The sucrose washing was sometimes aided by brief centrifugation at 100g.

2.2.5 Tobacco leaf protoplast isolation

For sterile preparations the precautions described in 2.3.1 were used. However, preliminary experiments at least, were carried out with care but not under aseptic conditions. Plants of 44-92 days old were selected, (this referring to Spring age, comparable stages of development being selected in other seasons.) Leaves from the middle portion of the plant were removed and allowed to become flaccid. Experience determined which leaves to select, according to appearance and texture. The more opalescent, grey and coarse leaves being unsuitable.

The lower epidermis was removed from the leaves using jeweller's forceps. The stripped region of the leaf was then cut out and placed stripped surface downwards on a petri dish of enzyme incubation medium.

Enzyme incubation media

Again levels of the components of the media were varied;

1.2-5% w/v Onozuka P1500 or

2-8% w/v Meicelase P or

0.2-2% w/v Purified Meicelase P

0.1-0.8 w/v Macerozyme

20-25% w/v sucrose or

10-16% w/v sorbitol

pH 5.8

Any additives and pretreatments are noted in section 3.5.3.

According to the enzyme incubation medium in use and the temperature, which varied from 20-40°C, the isolation was carried out in 2-20h.

Following isolation, protoplasts were separated from the incubation medium by one of two methods. Where sucrose was used, the protoplasts formed a narrow floating layer and could be pipetted off into a washing medium. However in sorbitol based media the protoplasts sank, therefore, after pouring through a wire mesh (1 mm) gauze to remove debris, the protoplasts were allowed to sink. The medium was pipetted off and replaced with washing medium. Several washings were necessary, aided if necessary by centrifugation.

Table 2.1

Summary of protoplast isolation conditions

(For clarity some details are omitted)

	Tissue	Preparation	Enzyme incubation medium	Incubation conditions			
				pH	Temperature	Time	Aseptic Static
1	<u>Oat</u> root tip	terminal 3mm excised	10% Onozuka P1500 5% Macerozyme 20% sucrose	Unadjusted	20°C	3-36h	+ +
2	<u>Maize</u> root tip	"	"	"	"	"	+ +
3 4	<u>Maize</u> coleoptile first leaf	dissected from seedling and finely chopped	3% Onozuka P3000 1% Macerozyme 9% sorbitol	"	"	6h	+ +
5	<u>Onion</u> root tip	As 1.	As 1.	"	"	3-36h	- +
6	<u>Onion</u> leaf	dissected from plant, pith removed, leaf sectioned and finely chopped	4-6% Onozuka P1500 or 3-6% Meicelase P 2% Macerozyme 2-20% sucrose	5.8	"	4-18h	- + or - (shaking at 60 r.p.m.)
7	<u>Tobacco</u> leaf	lower epidermis removed, leaf pieces cut out and floated on enzyme mixture	1.2-5% Onozuka P1500 or 2-8% Meicelase P or 0.2-2% Purified Meicelase P 0.1-0.8% Macerozyme 20-25% sucrose or 10-18% sorbitol	5.8	20-40°C	2-20h	+ and - +

2.3 CULTURE OF TOBACCO MESOPHYLL PROTOPLASTS AND SPONTANEOUS FUSION BODIES.

Tobacco leaves are highly contaminated with bacteria and fungi, thus obviating any culture, except very short term, when frequent media changing is necessary. For this reason all culture work involved observation of the following precautions:

2.3.1 Apparatus and Media Sterilization

Experiments were carried out in positive air flow sterile cabinets (Bassaire Ltd.) The interior surfaces were cleaned with a disinfectant solution, then alcohol, and the cabinet switched on for 1-2h prior to use. During use, surfaces were frequently swabbed with alcohol. Instruments were kept in methcol, being flamed just before use. Glassware, ceramic tiles and filters were sterilized by autoclaving at 15 psi for 15 min. Where available sterile disposable equipment was used.

All isolation, washing and culture media were used sterile. The enzyme incubation media were filtered with either Millipore or Sartorius (34 Göttingen Germany) filters using 0.45μ pore size membranes. Those with an appreciable solid content being centrifuged first. Other media were autoclaved at 15 psi for 15 min where possible or again filtered.

2.3.2 Surface sterilization of leaves

Two sterilization methods were used, being equally effective.

- a. Leaves were steeped for 30 sec in 70% v/v ethanol followed by 30 min in 10% v/v commercial bleach (Domestos, Lever Bros. Ltd.) then washed three times with sterile glass distilled water,

(latterly, sterile tap water).

b. Leaves were steeped for 2 min in 0.5% ^{w/v} dishwashing powder, 30 sec in 70% ethanol then 30 min in 5% sodium hypochlorite*. The leaves were then washed as above. (* 5% v/v of: 10-14% available chlorine)

2.3.3 Isolation and culture of protoplasts

The isolation procedures outlined in 2.2.5 were used. To ensure consistency of culture densities the protoplasts were then counted (see 2.4.1) and suitably diluted, the suspension being in the required culture medium (2.3.4).

Both liquid and solid cultures were prepared. For the former aliquots of protoplast suspension were placed in 100 ml Erlenmeyer flasks, sufficient to give a 3-4 mm depth. For solid culture, equal quantities of protoplast suspension and molten agar based medium were mixed in 12 mm petri dishes. (The medium was cooled to just above its solidification temperature.) (Note, to maintain constant culture densities, the protoplast suspensions were doubled in concentration before putting into solid culture.)

During culture the liquid preparations were supplemented to replace loss of medium. Any media changes were carried out by centrifugally compacting the protoplasts and replacing the supernatant. Initially liquid preparations were placed in a light cabinet with constant fluorescent lighting of 2000 lux and at a temperature of 22-25°C. At 5 days the cultures were transferred to a rotary shaker with a frequency of 100 r.p.m. under similar conditions of lighting and temperature.

Solid cultures were again placed in a light cabinet as above. The

dishes were turned upside down to prevent condensation effects.

Obviously these were not transferred to the shaker. Supplementation of medium was not necessary.

2.3.4 Culture media

Two media were commonly used, Modified White's medium (Lamport 1964) (Table 2.2) and TMS medium (Nagata and Tabeke, 1971) (Table 2.3). For the preparation of solid media 0.6% ^{w/v} agar was added to the required medium. Sucrose (2% w/v) was added to all media as a carbon source and either sucrose or sorbitol as a plasmolyticum.

Table 2.2

Modified White's Medium

$\text{Ca}(\text{NO}_3)_2$	200 mg/l
KCl	65
KNO_3	80
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	360
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	18.5
Na_2SO_4	200
$\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$	2.5
H_3BO_3	1.5
KI	0.75
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	4.5
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.5
Ca-D-pantothenate	0.1
Thiamine HCl	0.1
Cysteine HCl	1
2.4.D	3
6-BAP	1
Myo-inositol	100

Table 2.3TMS Medium

NH_4NO_3	825 mg/l
KNO_3	950
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	220
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1233
KH_2PO_4	680
Na_2EDTA	37.3
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8
H_3BO_3	6.2
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3
KI	0.83
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	0.030
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6
Myo-inositol	100
Thiamine HCl	1
I-NAA	3
6-BAP	1

2.4 QUANTITATIVE ASPECTS

2.4.1 Protoplast yields

To determine the yield of protoplasts per gramme of original leaf material, counts were done using the following method.

A Fuchs Rosenthal Counter, with a cover clearance of 0.2 mm was used, a normal haemocytometer being unsuitable. The protoplast suspension was concentrated to a few millilitres and a drop placed in the counter. Using the calibrated cover slip, the protoplasts in a given area, and therefore a given volume were counted. By extrapolation to the total preparation volume it was simple to work out the necessary dilution to convert the suspension to the required density.

2.4.2 Determination of extent of spontaneous fusion.

Several methods were used to try and accurately quantify spontaneous fusion:

a. Direct observation of fresh material.

At a certain stage of spontaneous fusion, it is possible to distinguish the number of constituent protoplasts in a fusion body. Nuclear counting using phase contrast microscopy, of fresh preparations was prevented in the tobacco mesophyll system by the presence of the numerous chloroplasts. Estimation of relative size gives some clue but this is best done from photographs.

b. Measurement of photographs.

The two major axes of the protoplasts and fusion bodies were measured.

c. Nuclear counting of stained preparations

Using the nuclear staining methods described in section 2.5.2, spontaneous fusion preparations were assessed for the degree of multinucleation.

d. Microdensitometry

Using the Vickers "M85 Scanning Microdensitometer", Feulgen stained preparations (2.5.2) were assessed for nuclear DNA content. (Walker and Richards, 1959). The microdensitometer was used under the following conditions: Objective x20 or x40, slit width 50, spot aperture 2, wavelength 552 nm.

2.5 LIGHT MICROSCOPY

2.5.1 Microscopes

Three photomicroscopes providing bright field, phase contrast and fluorescence facilities were used:

"Leitz Ortholux"

"Zeiss Photomicroscope"

"Nikon Microflex"

2.5.2 Staining techniques

Two nuclear stains, lacto-propionic orcein and Feulgen's stain, and a fluorescent brightener, Calcofluor White ST, to stain for cellulose were used with fresh protoplast preparations.

a. Lacto-propionic orcein. Orcein (1%)^{w/v} in a 50:50 v:v mixture of propionic and lactic acid acts as a stain fixative. A few drops were added to a protoplast suspension. After heating at

60°C for 2 min the preparation was carefully squashed. (Dyer, 1963).

b. Feulgen staining. Protoplasts were fixed in 4%^{v/v} neutral formalin for 4h, then washed in two changes of distilled water over 24h. The protoplasts, dried on to chrome-alum coated slides were hydrolysed in 5N hydrochloric acid for 50 min at room temperature, briefly washed then stained in Schiff's Reagent. (Commercial Feulgen Stain; G. T. Gurr, Searle Sci. Services) for 2h at room temperature. After washing in three changes of sulphite water (5 ml^{v/v} 10% aq. $\text{Na}_2\text{S}_2\text{O}_5$ + 5 ml 1N HCl + 100 ml distilled water) each for 10 min, the preparation was washed in distilled water, dehydrated through a series of alcohols to xylene and permanently mounted. (Modified from Conn, Darrow and Emmel, 1965).

c. Calcofluor. A 5% w/v solution of Calcofluor White ST in nutrient medium was added to solid (usually) media preparations of cultured protoplasts. These were viewed in the Leitz Ortholux photomicroscope. (Harrington and Roper, 1968).

One staining technique was used with embedded and sectioned material (2.5.3):-

d. Toluidine Blue. A millipore filtered solution of 1% w/v toluidine blue in absolute ethanol was added to sections mounted on slides. This was allowed to dry down on a hot plate. Excess stain was removed by washing in hot water. When dry the preparations were permanently mounted. (own method.)

2.6 ELECTRON MICROSCOPY

2.6.1 Electron microscopes

Three electron microscopes were used in this study, with the

following conditions of use:

GEC AEI "EM6B". Voltage 40-80 KV, using Meek Modification at 50 KV (Meek, 1967), and objective apertures of 25-70 μ . Magnification range x 1,500 to x 100,000.

GEC AEI "Corinth". Fixed voltage, objective apertures of 25-75 μ used. Magnification range x 600 to x 100,000.

Cambridge "Stereoscan". Voltage 20-40KV, used in the transmission mode.

2.6.2 Concentration of material

Due to their size and frequently paucity, protoplasts can be difficult to handle during fixation and embedding. A number of techniques were used to overcome this:

a. Centrifugal concentration. Protoplasts were retained in a conical medical centrifuge tube throughout the fixation procedures, at each stage being briefly centrifuged to compact the material. (After osmium fixation this was minimal since the protoplasts became considerably heavier.) Where protoplasts were plentiful they were transferred to a gelatin capsule (BDH size 00) for embedding, but where less so, shaped polypropylene capsules (eg TAAB) were used.

b. Microencapsulation. A thin glass capillary tube was dipped into hot 3% ^{1/1} agar. The capsule thus formed was partly pushed off the tube, dipped into the centrifugally concentrated protoplast preparation then sealed with agar, first at the distal end then cut off the capillary tube and sealed. The capsule was then treated as

a piece of tissue. (Henstra and Schmidt, 1970).

- c. Enclosure in gelatin. A similar technique to (b) but using an air bubble blown into a drop of gelatin to enclose the protoplasts. (B. W. W. Grout, personal communication)

2.6.3 Fixation and Embedding

Three fixatives were used, two commonly: glutaraldehyde followed by osmium tetroxide. The third, potassium permanganate (buffered 1.3%^{w/v} permanganate, (Pease, 1964)) was tried but with little success. Initial fixation was carried out by the addition of buffered glutaraldehyde to a protoplast suspension including a plasmolyticum. It was found that if sorbitol or mannitol rather than sucrose was used, poor general fixation, with particularly deleterious effects on the chloroplasts, was produced. (Withers 1973, Coutts, 1973). Post-fixation was with buffered osmium tetroxide.

Dehydration through a series of alcohols and staining with alcoholic uranyl acetate (see 2.6.4) preceded embedding, the former being carried out carefully as poor dehydration produced very unsatisfactory results. Two embedding media were used; Spurr Low Viscosity Resin, (Spurr, 1969) and a mixture of methacrylates and styrene. The latter was found to be most satisfactory in terms of compaction of material, specimen contrast and ease of cutting. Use of the resin was discontinued after trials. The mixture of methacrylate and styrene gave considerable flexibility in use, it being possible to alter the hardness of the medium by altering the ratio of butyl and methyl methacrylate. A generalised protocol for the fixation and embedding is given in table 2.4. (Modified from Mohr and Cocking, 1968).

The concentrations and times given refer to protoplast preparations,

with modifications in brackets for tissue pieces.

Table 2.4 Fixation and embedding procedure for protoplasts and tissues.

		<u>Temperature</u>	<u>Time</u>
Fixation	6% w/v glutaraldehyde in 0.025M phosphate buffer, pH7, plus 10-12% sucrose (1-6% glutaraldehyde in 0.025M phosphate buffer, pH7)	4°C	30 min-overnight (3h - overnight)
Washing	0.1M phosphate buffer, pH7 plus 10-12% sucrose (0.1M phosphate buffer pH7)	4°C	3 x 10 min. (3 x 30 min)
Postfixation	2% w/v OsO ₄ in 0.1M phosphate buffer plus 10-12% sucrose (0.5 - 1% OsO ₄ in 0.1M phosphate buffer)	4°C	30 min (2h)
Dehydration and staining during dehydration	30, 50, 70, 90% v/v ethanol	4°C	10 min each (30 min each)
	1% w/v uranyl acetate in absolute ethanol	20°C	30 min (2h)
	100% ethanol	20°C	2 x 30 min (2 x 2h)
Embedding	50 : 50,v : v,100% ethanol: embedding medium*	20°C	30 min (2h)
	Embedding medium	20°C	2 x 1h (2h then overnight)
Polymerization	Transfer to fresh embedding medium in capsules	56°C	40h

*Embedding medium: 7 parts methacrylate**
 3 parts styrene**
 1% w/v benzoyl peroxide
 CaSO₄ (anhydrous) to dehydrate(** stabilizers present)

The ratio of butyl methacrylate to methyl methacrylate is varied to give varying degrees of hardness, methyl tending to harden the medium. Routinely, equal parts of the two methacrylates were used or occasionally

4 parts of butyl to 3 methyl for a softer medium.

2.6.4 Sectioning

Two ultramicrotomes, the Porter Blum MT2 and the Cambridge - Huxley, were used. Sections were cut using glass knives carrying copper troughs. Cutting faces were selected by firstly cutting thick sections and viewing in the light microscope. Sections of three ranges of thickness were cut, gold silver (50 - 90 nm) for normal voltage electron microscopy, semi thick (250 - 750 nm) for high voltage and scanning electron microscopy and thick (500 - 1000 nm) for light microscopy.

Thin and semi-thick sections were expanded with trichloroethylene, where possible, and mounted on formvar coated copper grids. (Athene, Hexagonal or New 200). Thick sections were collected, unexpanded in a wire loop, placed on a slide and expanded by gentle heating.

2.6.5 Staining techniques

a. General staining. Routinely, preparations were stained during dehydration with alcoholic uranyl acetate (1% w/v in absolute ethanol), in preference to using it as a post stain, then post stained with lead citrate. (Reynolds, 1963). The latter gave good contrast penetrating even 500 nm sections. It was applied for times varying between 5 and 15 min depending on section thickness. Staining was carried out in a carbon dioxide free atmosphere and was followed by copious washing.

b. Specific stains. Two were used to identify pectins and plasmalemma:

Pectin stain. (Modified from Albersheim, Mühlethaler and Frey-Wyssling, 1960). Glutaraldehyde fixed tissue pieces were washed in 0.05M Phosphate buffer, pH7, and dehydrated to 60% v/v ethanol. The tissue was placed for 30 min in a mixture of equal parts of 14% w/v crystalline hydroxylamine hydrochloride in 60% ethanol and 14% w/v sodium hydroxide in 60% ethanol. An equal volume of one part hydrochloric acid (specific gravity 1.88) and two parts 95% v/v ethanol was added for a further 30 min. The tissue was transferred for one hour to 2.5% w/v ferric chloride in 0.1N HCl in 60% ethanol. After washing in 60% ethanol the tissue was dehydrated and fixed as described in table 2.4.

Plasmalemma stain. (Modified from Roland, Lembi and Morré, 1972). Thin sections, mounted on formvar coated grids, were placed on a drop of 1% v/v periodic acid for 30 min, washed then transferred to a drop of freshly prepared 1% w/v phospho-tungstic acid in 10% v/v chromic acid for 10-20 min. The sections were again washed, then air dried. Formvar coating was essential to protect the copper grids and ensured section adhesion. Unmounted sections (as used by Roland et al.) would have avoided this problem but were difficult to handle.

Summary
(Chapter Two)

The isolation of protoplasts and spontaneous fusion bodies from a number of tissues is described. Details of plant growth conditions, tissue sterilization, enzyme preparations and enzyme incubation conditions are given.

Techniques for the culture of tobacco mesophyll protoplasts and spontaneous fusion bodies, on liquid and solid media are described, with details of the culture media used.

Several methods, using size estimation and staining techniques, for the assessment of the level of spontaneous fusion in a preparation are described.

Details of light and electron microscopical techniques employed are given, including aspects of protoplast handling, fixation, staining and embedding techniques.

Some of the methods relating to protoplast isolation are summarised in Table 2.1; the electron microscopical (preparative) techniques are summarised in Table 2.4

CHAPTER THREE

THE SPONTANEOUS FUSION OF PROTOPLASTS

RESULTS

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3.1 THE OAT ROOT SYSTEM

This investigation included the study of the anatomy of the untreated oat root and the pattern of enzyme digestion of the tissues in terms of the gross changes and fine-structural changes occurring. These latter aspects are related back to the original tissue structure.

3.1.1 The structure of the oat root

The region of the oat root studied extends from the root cap proximally to include 1 - 2 cm of stele. Figure 3.1 drawn from thick sections of embedded material, viewed in the light microscope, shows the regional differentiation of the tissues. The root cap is of irregularly shaped, thick walled cells. Adjoining these in the extreme root tip itself is the quiescent centre. Laterally the root tip is limited by a thick walled epidermis and underlying sub-epidermal layer of cells.

The stele has ordered longitudinal lines of cells, those towards the centre tending to be irregular or elongated longitudinally and the more peripheral ones broader in the transverse plane. The central stele cells furthest away from the root tip show the beginnings of vascular organization.

Comparative anatomical studies with the scanning electron microscope in the transmission mode and transmission electron microscope at high voltage (80 KV) were carried out. The latter proved to be the more satisfactory. Figure 3.2, taken in the scanning electron microscope, shows peripheral stele cells. Little detailed structure can be made out apart from cellular dimensions, wall thickness and the tendency for the cells to plasmolyze away from the longitudinal walls. More detail was supplied using thick sections in the transmission electron microscope.

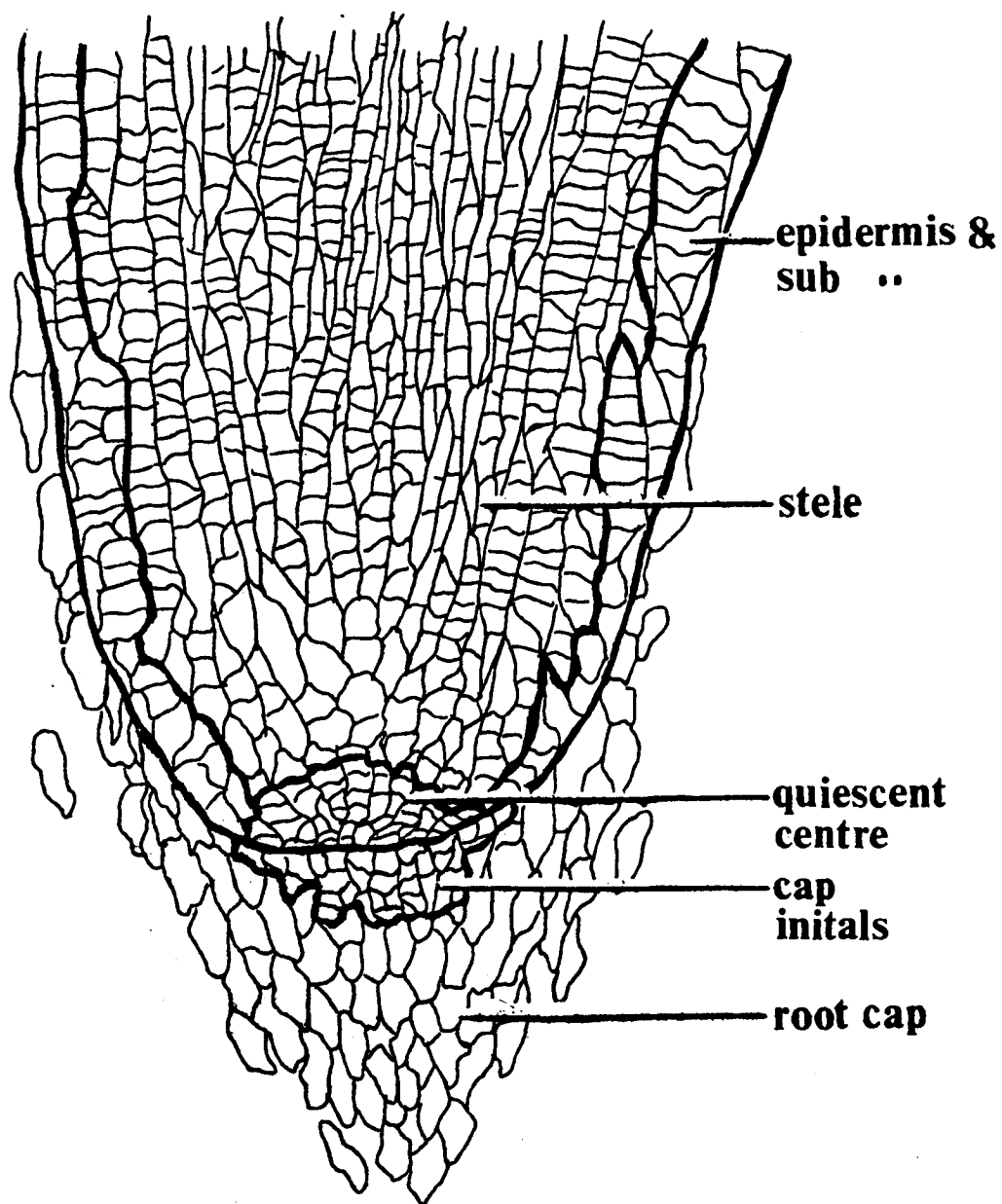


Figure 3.1

Median longitudinal section of an *Avena sativa* seedling root tip. The figure is redrawn from sections of embedded material. The length of root tip and root cap represented is approximately 2 mm. The regions of the root tip are identified.

Two highly distinct regions are shown in Figures 3.3 and 3.4, from the peripheral stele and central stele regions respectively. In the former, the cell proportions, wall thickness and plasmolytic tendencies are seen as in Figure 3.2 but somewhat more clearly. In contrast, the cells in Figure 3.4 are more irregular in form, plasmolyzing generally, with the suggestion of regions of greater cytoplasmic continuity - fields of plasmodesmata (arrowed). The degree of vacuolation is greater than in the peripheral stele cells, this tendency increases with distance from the root tip, with the exception of the quiescent centre cells which are more vacuolate than adjacent stele cells.

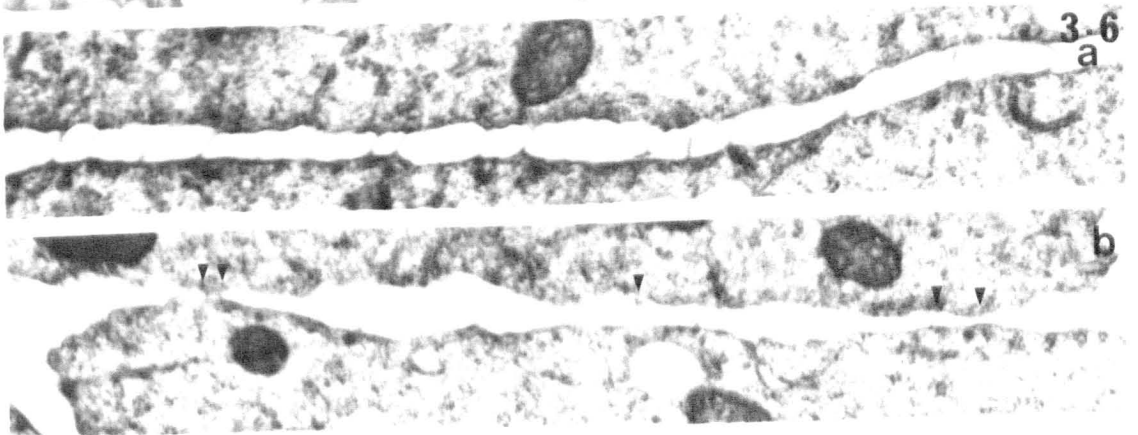
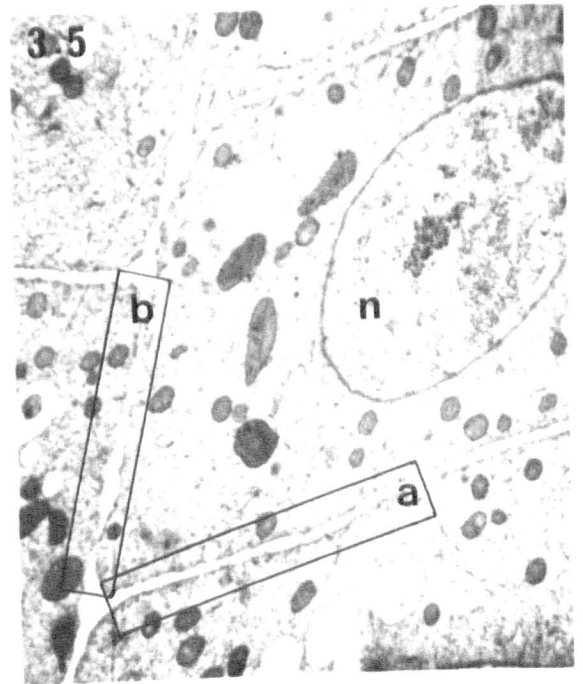
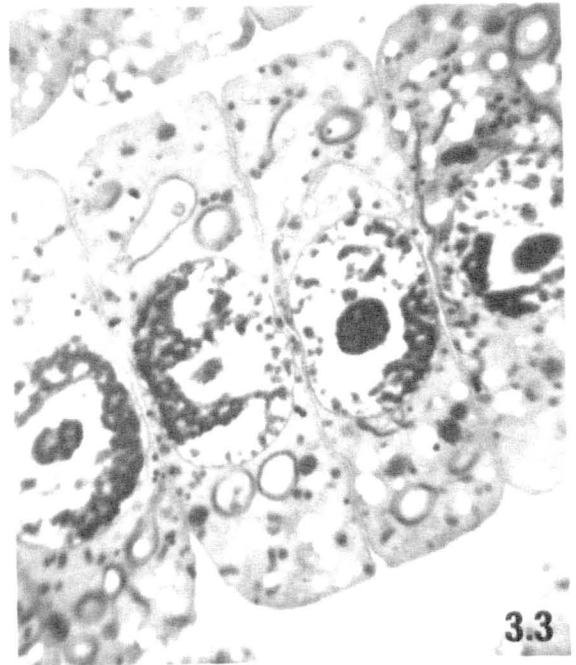
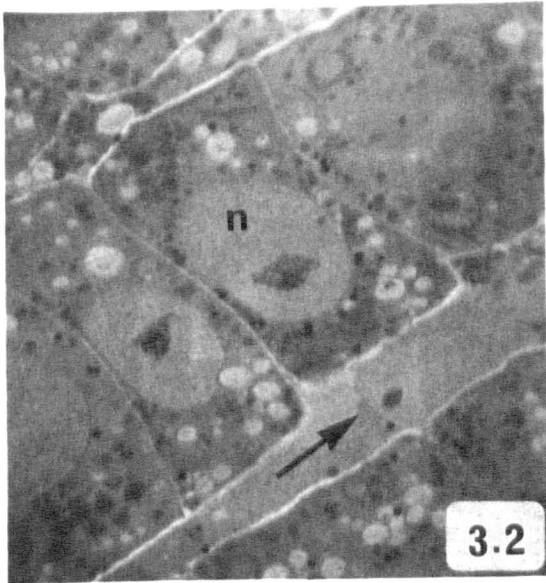
Similar investigations applied to the remaining regions of the root tip show that the epidermis and sub-epidermis are thick walled, with a low degree of vacuolation and correspondingly little plasmolysis and the root cap cells are very thick walled, highly vacuolate and plasmolyse extremely.

Although providing a pleasing image, with good contrast and moderate resolution, the thick sections provided little fine structural detail. Thin sectioning was therefore employed. Figure 3.5 shows a region not unlike to that in Figures 3.2 and 3.3 but slightly younger with a consequent similarity in wall thickness on transverse and longitudinal faces. However there is a clear distinction between the two walls in their plasmodesmatal distribution. Figures 3.6a and b show high power views of regions of the transverse and longitudinal walls respectively. The plasmodesmata are more or less evenly distributed on the former but are less numerous and are grouped on the latter wall. In isolation, this section gives a poor quantitative comparison, it being necessary to use serial sections.

An analysis of plasmodesmatal distribution was carried out to

The structure of the oat root tip

- Figure 3.2 Thick(1μ ,unstained) section viewed in the 'Stereoscan' operating at 30KV. The peripheral stele cells form longitudinal rows,separated by thick walls and a broad plasmolysis space(arrow). Within each cell, the central nucleus, small organelles and cytoplasmic vesicles can be seen.(n=nucleus) x 1,000
- Figure 3.3 Thick (0.6μ ,unstained) section viewed in EM6B operating at 80KV. The peripheral stele cells resemble those in the figure above, but the section gives better contrast. x 1,000
- Figure 3.4 Central stele cells.(Section details as Figure 3.3). The cells have larger vesicles/vacuoles. Plasmolysis occurs on all walls but irregularly. Arrow indicate likely fields of plasmodesmata. x 1,000
- Figure 3.5 Young stele cells, shown in thin section(stained). In this region, the transverse and longitudinal walls are equally well developed but the plasmodesmata distribution does differ: x 2,000
- Figures 3.6a and b Enlarged,boxed regions of Figure 3.5. In Figure 3.6a, the many plasmodesmata on this transverse wall can be seen clearly. On the longitudinal wall, however,(Fig. 3.6b) they are fewer in number and difficult to see in the section.Arrows mark their location. x 6,000



parallel work done by Barlow and Juniper (1969) on the maize root tip. By counting the plasmodesmata on transverse and longitudinal walls in consecutive sections, a ratio of plasmodesmata per unit area of wall could be established. Assuming the area of wall represented in an electron micrograph to be a function of wall length, not the square of wall length (since the section is of finite thickness and less than the depth of field of the electron beam), the ratio can be applied to the plasmodesmatal distribution per unit area also.

Table 3.1.1 shows the results of the analysis, with comparable values for the maize root tip derived from the data of Barlow and Juniper (1969).

Table 3.1.1 Ratio of plasmodesmata per unit area on transverse and longitudinal walls in different regions of the oat root tip. Data for maize root tip given in brackets.
(Replicates insufficient to give standard deviations.)

Cap	8.37 (10.3 - 11.4)
Cap initials	4.25 (2.85)
Quiescent centre	1.35 (0.96)
Epidermis, sub epidermis	2.58
Stele, central, 1st 10 rows	1.72 (1.65)
Stele, central, 11th - 20th rows	3.18
Stele, central, 21st row - cut end	1.77
Stele, peripheral	5.95

Thus there is a predominance of plasmodesmatal connexions across the transverse walls, particularly in the root cap and peripheral stele regions. Where the wall areas on longitudinal and transverse faces differ greatly, such as in the peripheral stele cells, the ratio of plasmodesmata over the total wall area is even greater.

As already pointed out, an associated variable in the system is

wall thickness. No quantitative data were collected on this but generally longitudinal walls tend to be thicker and of a more irregular thickness than horizontal walls in any one area. The differences became less marked as the cells matured.

The information gained on the anatomy of the root tip is used later where the pattern of spontaneous fusion within the root tip tissues is considered.

3.1.2 Enzyme digestion and protoplast release: light microscopic observations.

Simply to produce protoplasts and fusion bodies, sagittally cut root tips were used. These were taken after periods of enzyme digestion extending from 3 - 36h and either teased or gently squashed to break up the tissues.

Up to 12h, little more than a few single protoplasts and isolated vacuoles were produced. By 18h, the tissues yielded more to treatment, releasing a mixture of cells, protoplasts and fusion bodies. The cells tended to remain in lines (Fig. 3.7). It was occasionally difficult to determine whether complete wall digestion had in fact occurred. Figure 3.8 shows a line of cells which clearly have intact walls surrounding and dividing them. However in Figure 3.9 (arrow) a similar structure is seen which could possibly be classified as a fusion body. In the same micrograph (double arrows) there is a line of cells which by their distortion at the cell interfaces show an obvious attachment to transverse dividing walls, concave plasmolysis drawing the protoplasts away from the longitudinal walls. In other cases (Fig. 3.10) the extent of plasmolysis isolates each protoplast clearly revealing the dividing wall.

Enzyme digestion of the oat root tip

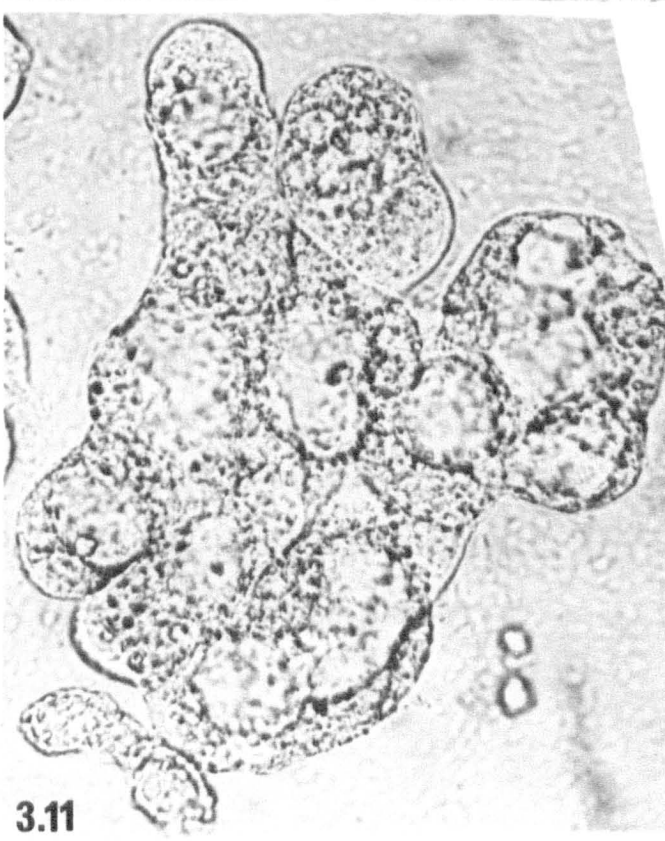
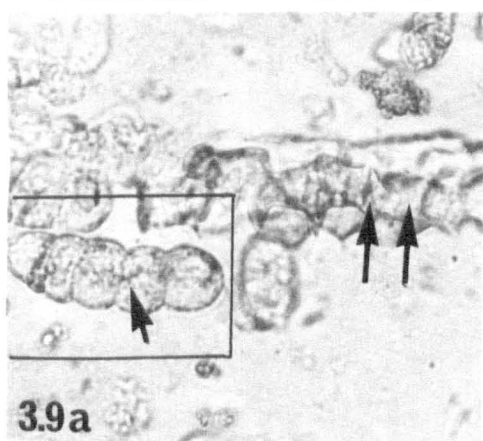
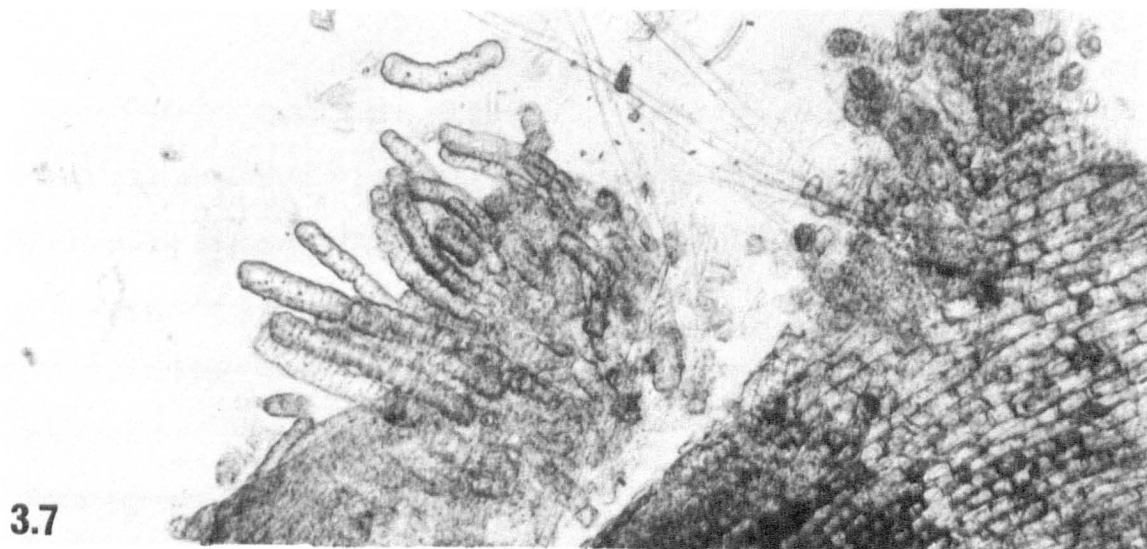
Figure 3.7 After 18h digestion rows of cells and linear fusion
bodies are released. x 200

Figure 3.8 A row of stele cells. The walls are still present and
the cell interfaces are clear. phase contrast
x 1000

Figure 3.9a A possible fusion body (arrowed) can be seen. (Enlarged in
Figure 3.9b). The double arrows indicate a row of
concavely plasmolysed cells. The walls are obviously
still present. a x 300
b x 600

Figure 3.10 Here the protoplasts are completely plasmolysed
away from the cell walls. Little digestion has occurred.
phase contrast
x 500

Figure 3.11 (Kindly supplied by Dr. J.B.Power) A large spontaneous
fusion body. The linear arrangement of the contributing
protoplasts is still evident to some extent, but
the fusion body is becoming generally more rounded,
indicating that wall digestion is complete.
x 600



Longer enzyme treatment, beyond 18h, causes further tissue digestion and protoplast release. The occurrence of lines of partially digested cells changes little but more fusion bodies and protoplasts are released, the former assuming more rounded shapes (Fig. 3.11) clearly indicating that the limitations imposed by the cell walls have been removed.

Some preparations were treated with 3.5% w/v sodium nitrate (isotonic with 20% w/v sucrose) for 30 min following 24h of enzyme treatment. No increase in fusion was detected and in some cases there was a deleterious effect on the protoplasts, bursting occurring.

3.1.3 Enzyme digestion and protoplast release: electron microscopic observations.

Note: Here, whole root tips were used and therefore by "release" it is meant that the protoplasts (or fusion bodies) were freed of their cell walls and released within the root tip structure rather than from it.

Whole root tips treated for 3 - 36h at three hourly intervals were fixed and embedded without disturbing the tissues. Entire longitudinal thin sections were cut, corresponding to that shown in Figure 3.1 and the tissues were "mapped" noting the extent of plasmolysis, wall digestion and fusion. To fully understand the descriptions given, it is necessary to first explain in detail the changes taking place without specific reference to their location. The observations are taken both from the whole root tip sections and where necessary from thinner smaller sections.

A considerable amount of tissue damage occurs, some obviously due to mechanical causes and fixation, especially at the cut end, but other damage needs further explanation. Plasmolysis invariably occurs, to

what extent and to what degree it is accompanied by wall digestion, depends on the region in question. Figure 3.12 shows a region where wall digestion was virtually nil, but the protoplasts have contracted away from the wall, breaking the field of plasmodesmata and leaving small fragments of cytoplasm behind. In contrast, a younger region shows a less extreme reaction to plasmolysis (Fig. 3.13). One plasmalemma is intact whilst the other is damaged, leaving a plasmodesma (arrow) still attached to the little digested wall. Again fragments of cytoplasm lie between the protoplasts and the wall. Observation of serial sections shows that these take both a rounded and cylindrical form, often being attached at one end to either the plasmalemma or wall.

It would appear that some membrane repair takes place, but much of the plasmolysis damage remains and following wall digestion deplasmolysis is seen to occur. Cytoplasmic projections, apparently invaginating from one protoplast into an adjacent damaged protoplast were common (Figs. 3.14 and 3.15). In the latter micrograph, the damage is obvious whereas in the former, the membrane damage occurs some distance away, but cytoplasmic contents can be seen lying between the two protoplasts. Considering the extent of this leakage it is surprising that organellar damage was so slight. In Figures 3.16 and 3.17, plastids and mitochondria from damaged cells remain intact and well fixed, although they lie in an area of highly dispersed cytoplasm.

Frequently the undamaged protoplasts in such an area have plasmodesmatal connexions to fairly large, membrane bound cytoplasmic fragments (Figs. 3.16, 3.17 arrows) suggesting considerable healing properties of the plasmalemma reducing considerably the vulnerability of

Enzyme digestion of the oat root tip

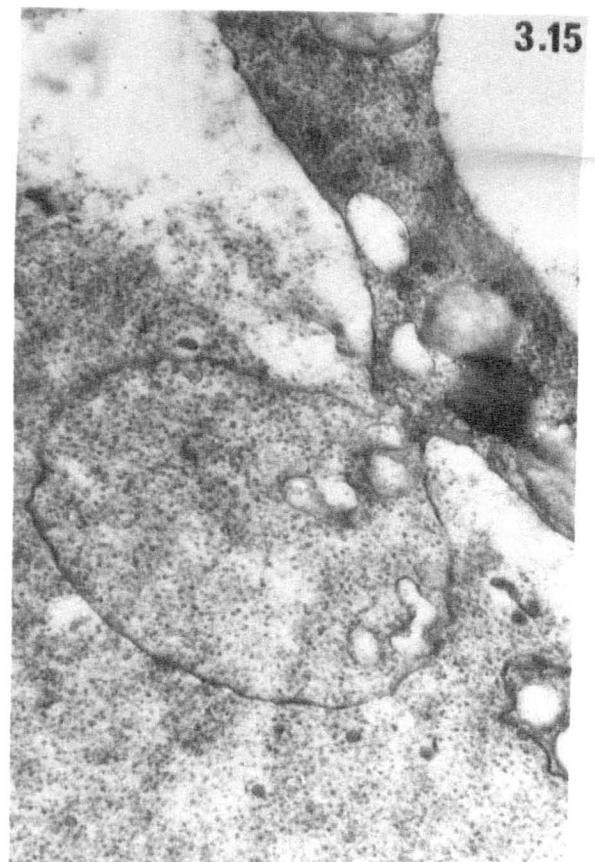
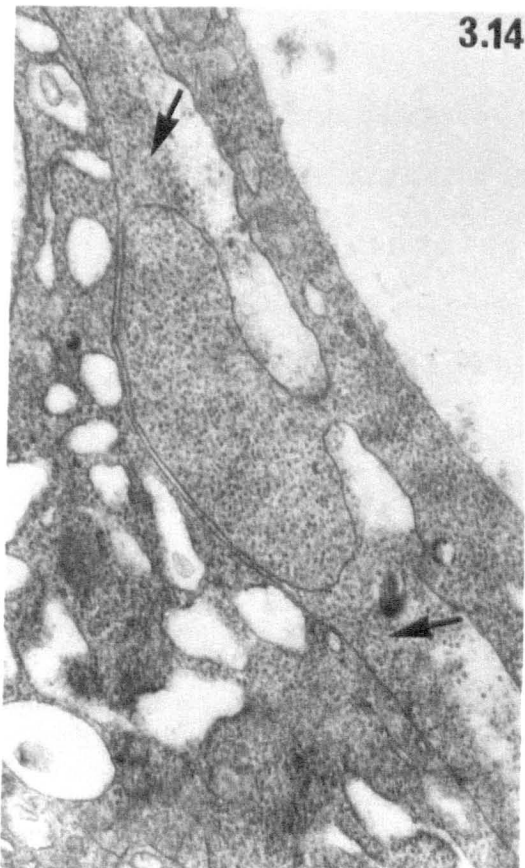
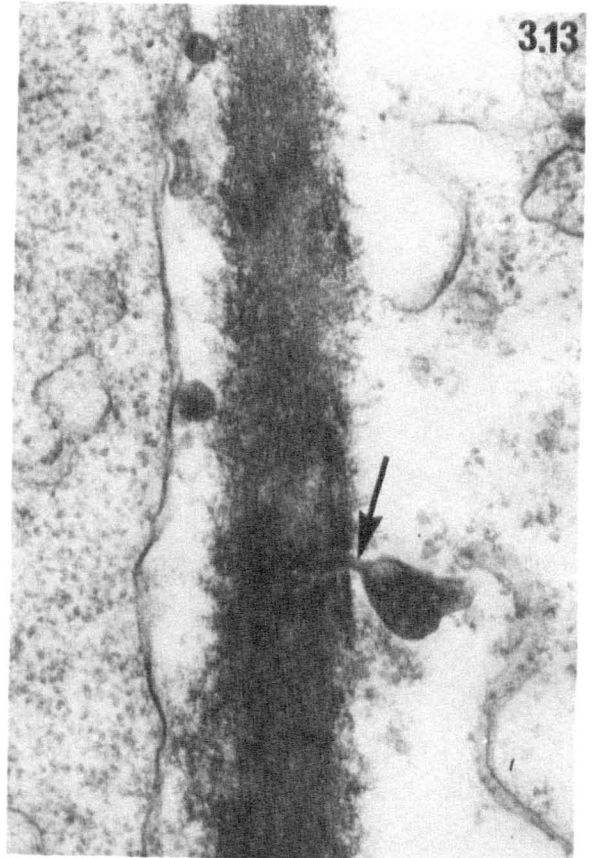
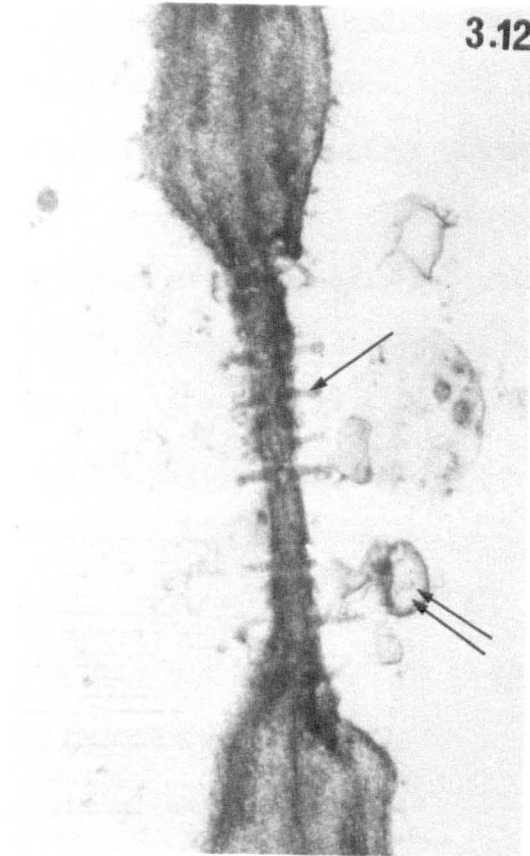
Damage occurring during plasmolysis and wall digestion

Figure 3.12 Little wall digestion has occurred. The protoplasts have completely withdrawn from the wall. Broken plasmodesmata (arrow) and cytoplasmic fragments (double arrows) can be seen. x 60,000

Figure 3.13 A less heavily plasmolysed, younger region of the root tip. The protoplast to the right is damaged, a plasmodesma (arrowed) with some attached cytoplasm, retains its position within the cell wall. x 80,000

Figure 3.14 Wall digestion has occurred in this region. A projection has formed from one protoplast, extending towards the neighbouring one. Cytoplasmic contents lie between the protoplasts. (arrowed) x 35,000

Figure 3.15 As Figure 3.14, however here one of the protoplasts invaginates into the other, obviously damaged, one. x 40,000



the tissue mass in general to damage. This phenomenon could explain the apparent invaginations shown in Figures 3.14 and 3.15 although the "necks" of the invaginations do not have a plasmodesma like appearance. As will become apparent plasmodesmatal expansion could resolve this.

Stacks of endoplasmic reticulum (e.r.), mainly rough, are commonly seen. In Figure 3.16 a stack of e.r. links the nucleus to the plasmodesmata on the cell periphery. It is noted that e.r. stacks were not exclusive to damaged tissue however.

Where damage is extensive, large masses of apparently fused cytoplasm can form (Fig. 3.18). Within such an area, transverse cell interfaces remain distinct (arrows) with associated groups of organelles, whereas the longitudinal interfaces are difficult to locate.

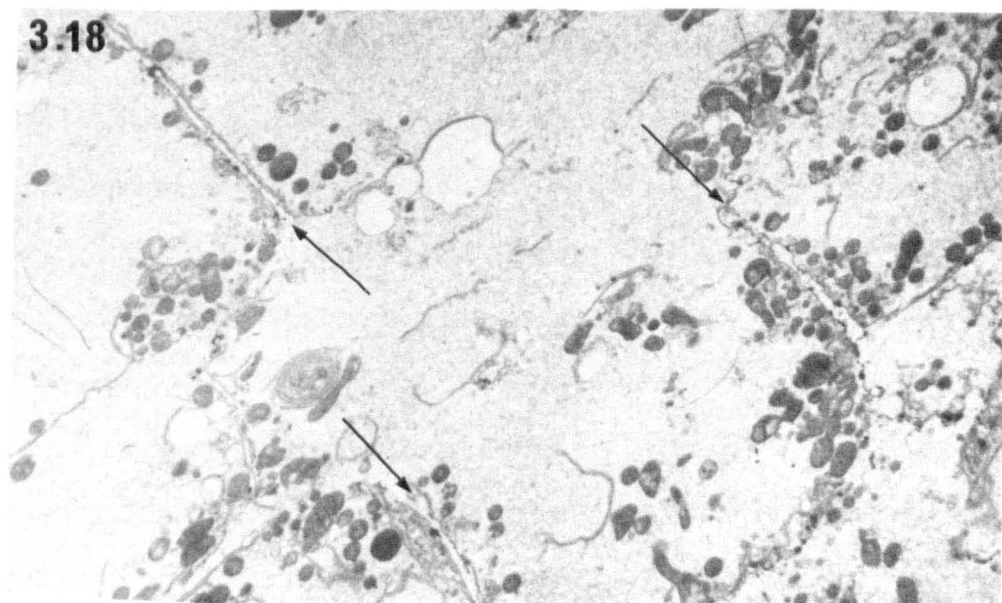
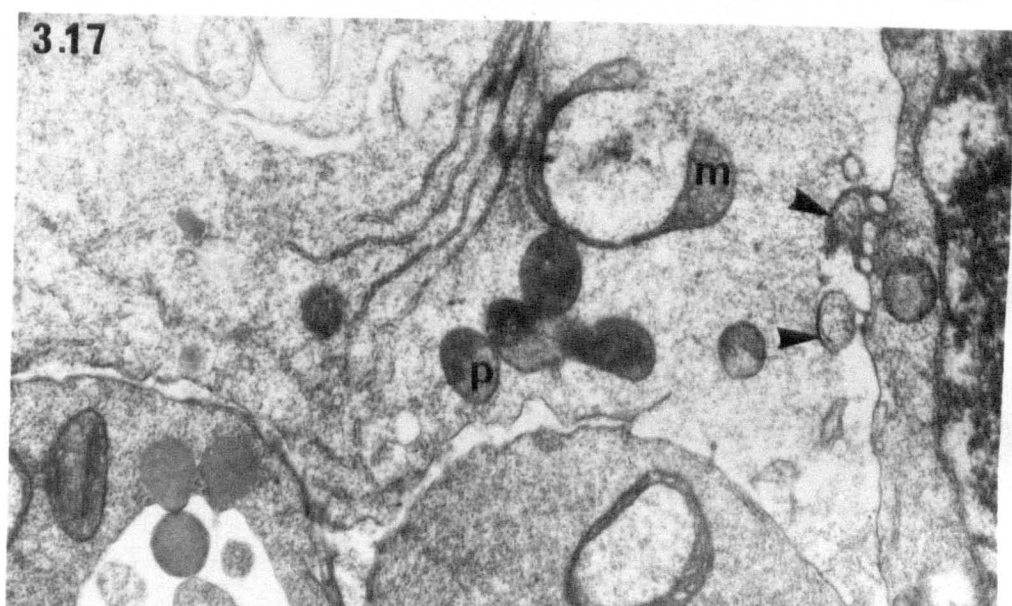
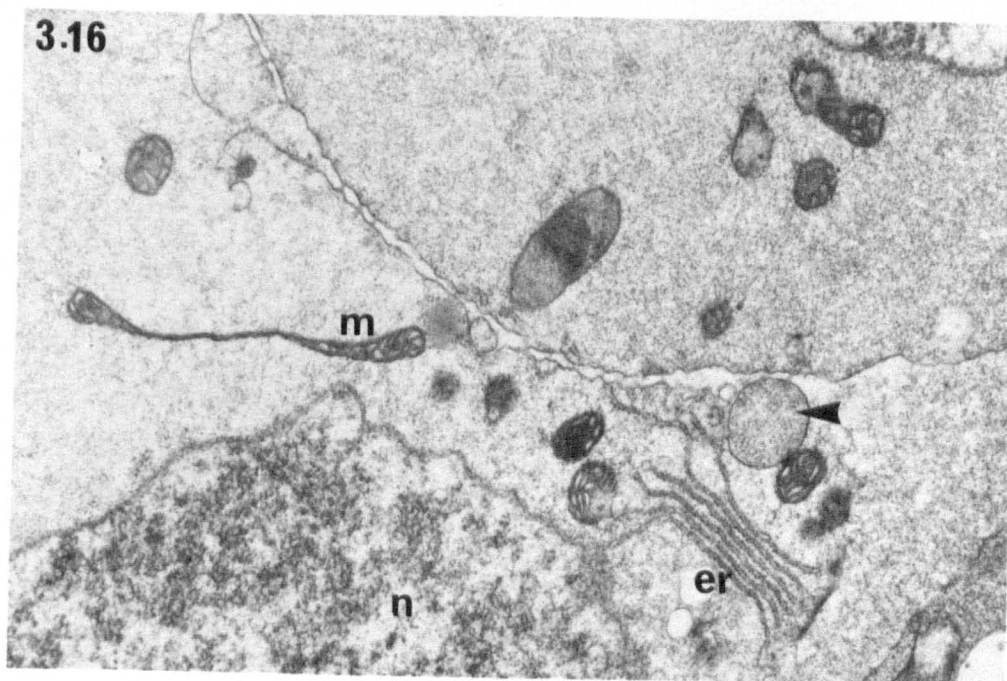
In the majority of the root tissues other modifications take place to accommodate the changes brought about by plasmolysis and wall digestion. Plasmodesmata form numerous links between adjacent cells, representing large areas of cellular continuity (Fig. 3.19). Details of the plasmodesmatal structure are indistinct in the untreated tissue. The plasmodesmata join the plasmalemma more or less at right angles, showing that in the uniplasmolysed state there is little tension across the plasmodesmata. Although their length varies, the plasmodesmata have a fairly consistent width of 40 nm .

As plasmolysis and wall digestion ensue and the plasmalemmae withdraw, some plasmodesmata break as mentioned, but many remain intact although showing signs of stress. The ends of the plasmodesmata broaden, their points of junction with the plasmalemmae becoming less clear. (Figs. 3.20 and 3.21). There were invariably e.r. connexions with the plasmodesmata, shown particularly clearly in the thicker section of Figure 20. In Figure 3.21 wall digestion is more or less complete and

Enzyme digestion of the oat root tip

Damage occurring during plasmolysis and wall digestion

- Figures 3.16 and 3.17 Plastids (p), mitochondria (m) and e.r. lie in areas of highly dispersed cytoplasm, which result from rupture of the protoplast plasmalemmae. In Figure 3.16, the e.r. stacks join the nucleus (n) to remnants of the plasmalemma. The surrounding, undamaged protoplasts are connected, by plasmodesmata, to membrane bound cytoplasmic fragments on the periphery of the damaged regions. Figure 3.16 x 15,000
Figure 3.17 x 17,500
- Figure 3.18 A large area of damaged cytoplasm. Organelles are grouped mainly near the remnants of the transverse cell interfaces (arrowed). It is difficult to trace the longitudinal cell interfaces. Figure 3.18 x 3,000



the plasmodesmata show signs of a general increase in width to c.50nm . Their structure becomes clearer and it is possible to visualise the desmotubule (Robards, 1968) running through the centre of the plasmodesma. In the semi-transverse section of Figure 3.22 this is again seen (arrows) (Plasmodesmatal ultrastructure and the relationship of the e.r. to plasmodesmata are discussed fully in Appendix 1.)

Plasmodesmata are frequently located close together. In the digested tissue such pairs of plasmodesmata often appear, drawn together (Fig. 3.23, right) with a common e.r. membrane joining them at one end. Also in this Figure (left) a broad (c.70nm) plasmodesma, lacking a desmotubule is seen. Further observation clarifies this. It appears that when the plasmodesmata expand beyond approximately 60nm , the desmotubule remains attached to one side whilst the plasmalemma detaches on the other side allowing expansion to continue and ribosomes to enter the newly created space. (Figs. 3.24 and 3.26). The plasmodesma in Figure 3.23 would therefore appear to be cut such that the section misses the laterally placed desmotubule.

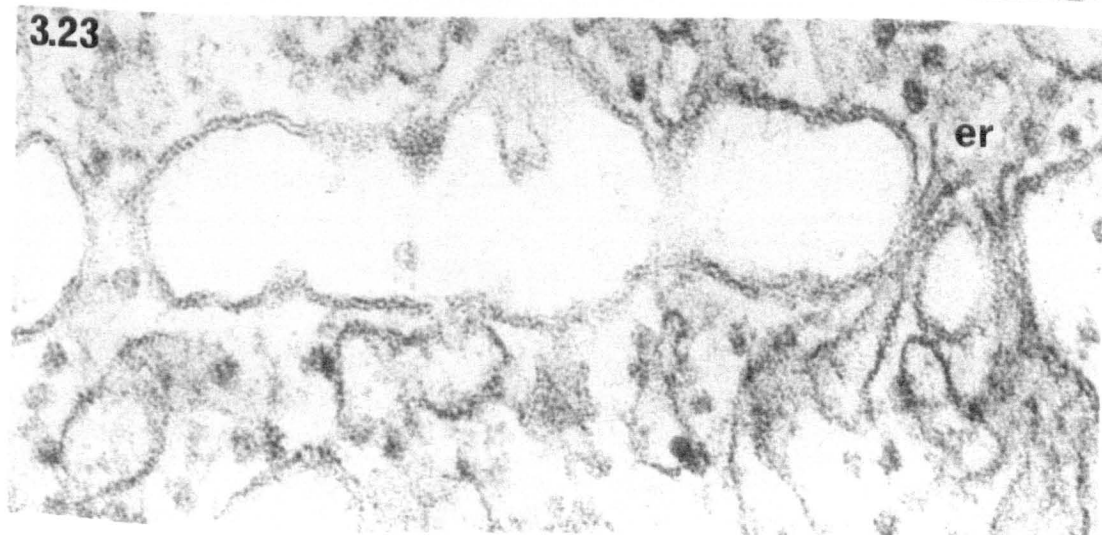
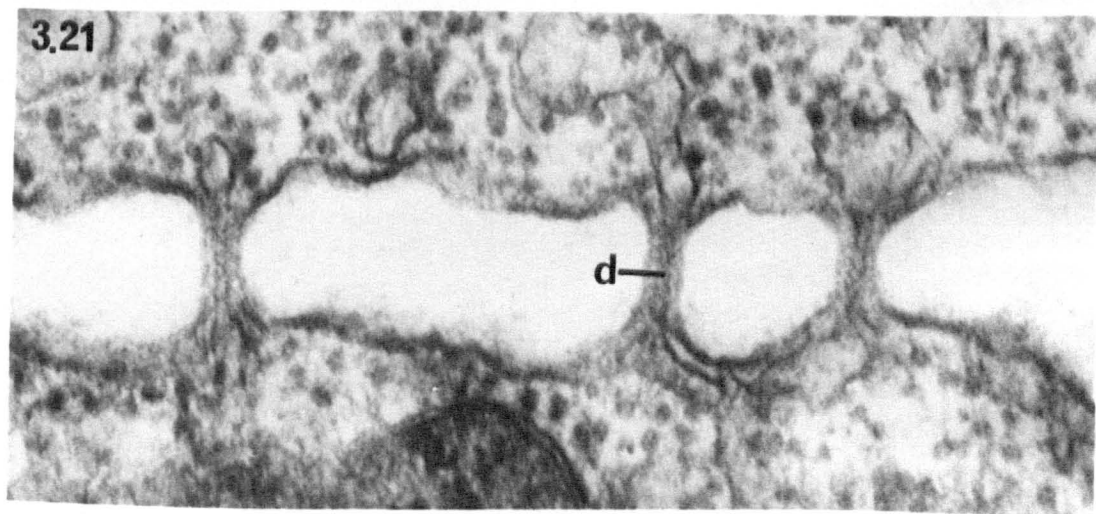
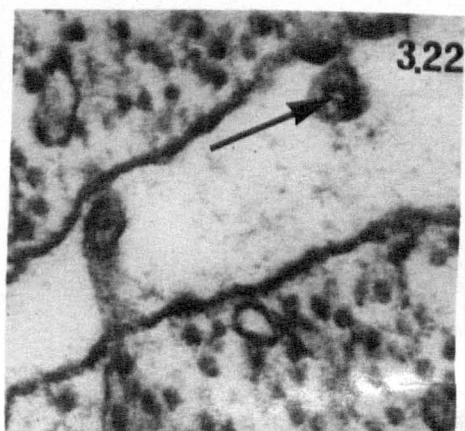
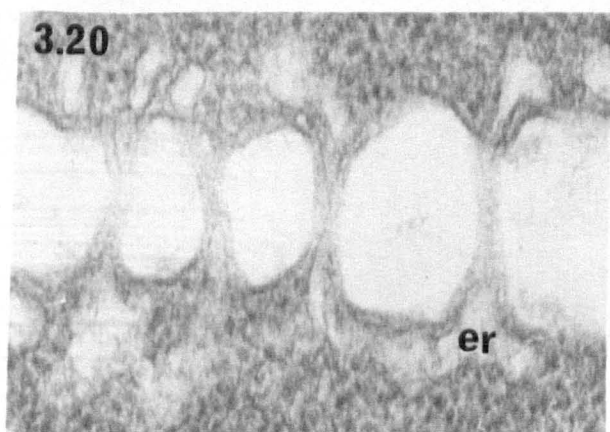
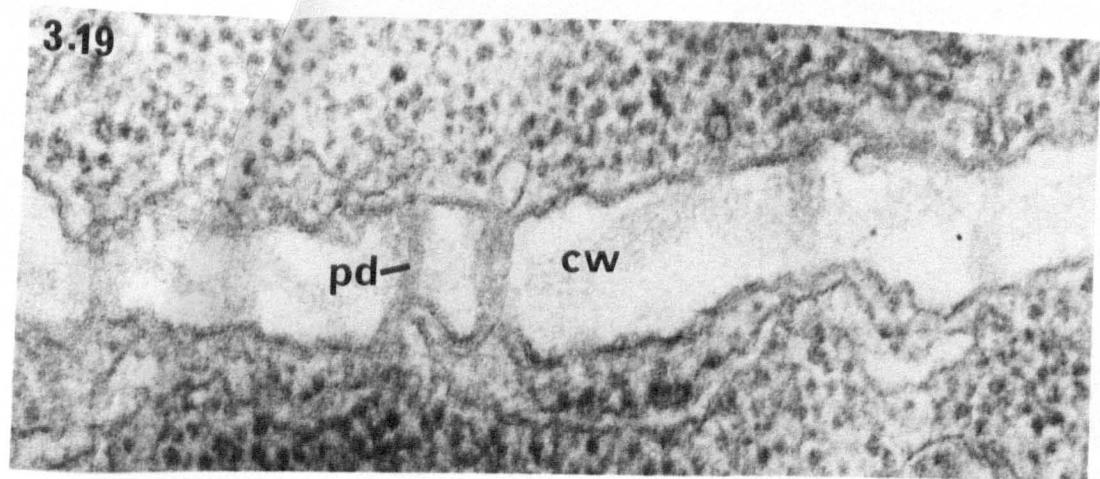
In contrast, Figure 3.27 shows a broad, membrane like structure (arrow) within the expanded plasmodesma. This possibly represents a displaced e.r. membrane rather than providing evidence for expansion of the desmotubule. Figure 3.25 shows several plasmodesmata with associated stacked rough e.r. continued in the adjacent, lower, protoplast. Figure 3.31 shows similar structures apparently bridging adjacent protoplasts, the desmotubules not in evidence. The protoplast connexions are here very broad. By this stage the plasmodesmatal stretching first evident at the onset of plasmolysis (Fig. 3.20) appears to be reversing.

The grouping of organelles near a transverse cell interface shown in Figure 3.18 is seen again. Plastids and mitochondria lie close to

Enzyme digestion of the oat root tip

plasmodesmatal changes

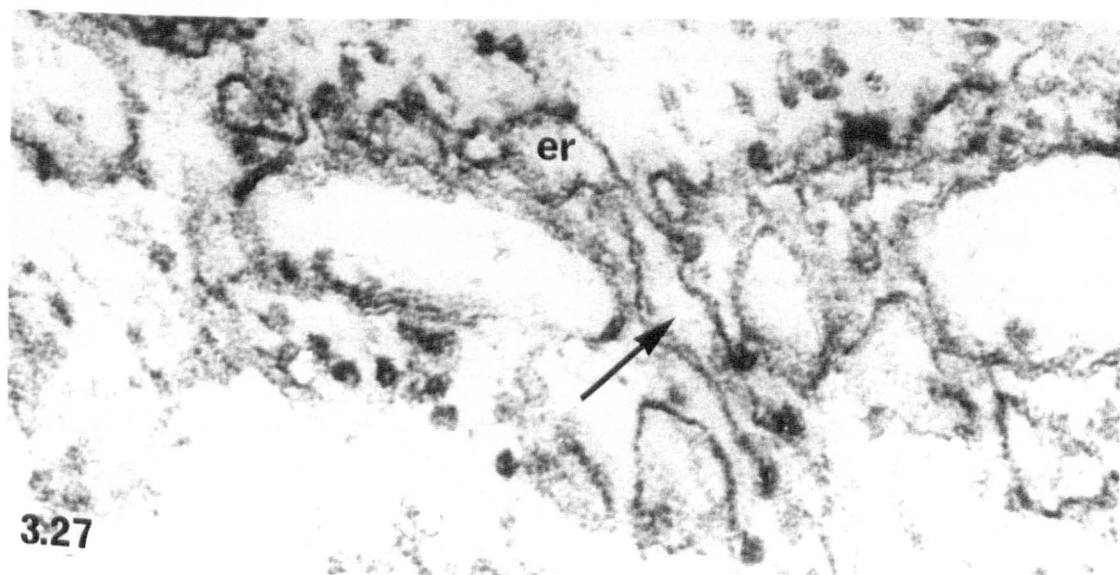
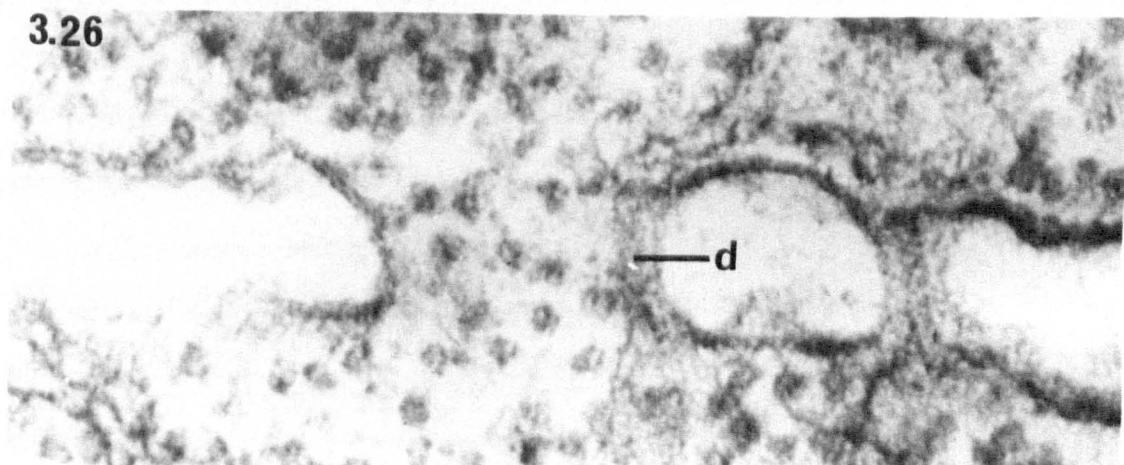
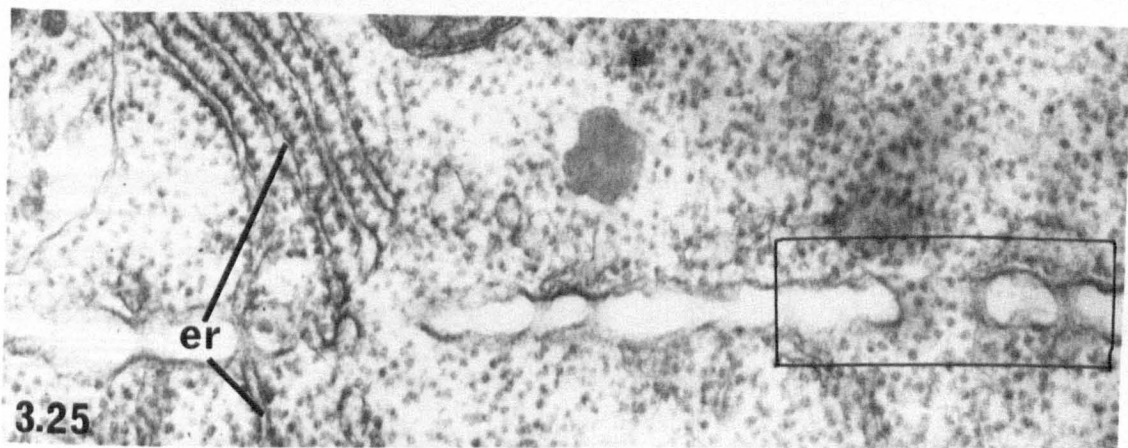
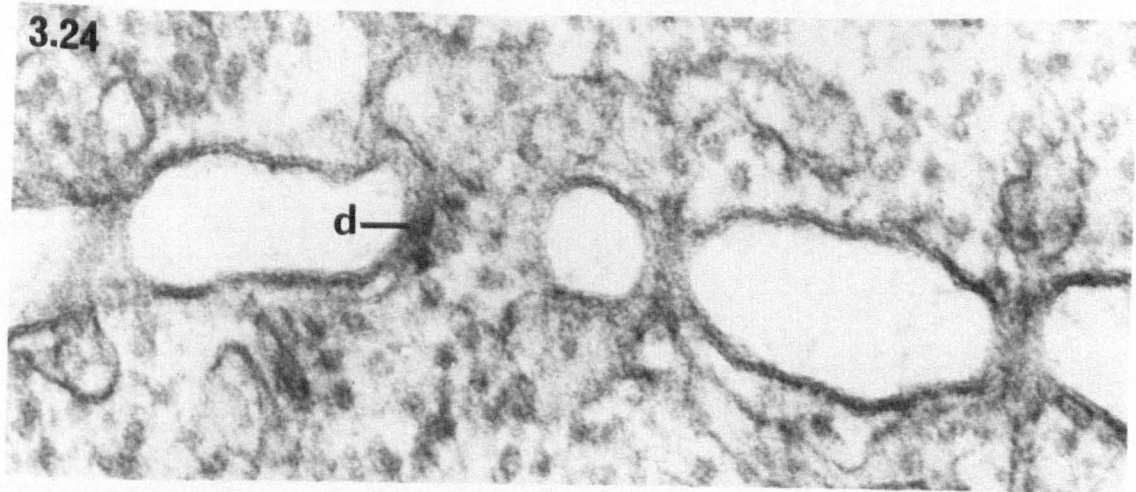
- Figure 3.19 The untreated root tip. Plasmodesmata traverse the cell wall (cw), linking adjacent cells.(pd= plasmodesma.) x 98,000
- Figure 3.20 Some wall digestion has occurred. Plasmolysis has withdrawn the plasmalemmae, thus stretching the plasmodesmata, but they remain intact. The e.r. membranæ at the end of the plasmodesmata can be seen clearly. x 85,000
- Figure 3.21 Wall digestion is complete. The plasmodesmata appear slightly clearer and broader than in the untreated tissue. The desmotubule is clearly seen. (d=desmotubule) x 120,000
- Figure 3.22 A semi-transverse section shows the desmotubule (arrow) located centrally within the plasmodesma. x 110,000
- Figure 3.23 Left; a broad (70nm) plasmodesma appears to be lacking a desmotubule. Right; a common e.r. membrane joins the desmotubules of the two plasmodesmata. x 150,000



Enzyme digestion of the oat root tip

plasmodesmatal changes

- Figure 3.24 Plasmodesmatal expansion is occurring. Ribosomes have entered the broad plasmodesma. The desmotubule is displaced to one side. x 140,000
- Figure 3.25 Left; stacked e.r. is associated with a group of plasmodesmata. Right; enlarged in Figure 3.26: x 50,000
- Figure 3.26 Similar to Figure 3.24. Again the desmotubule is laterally placed. x 155,000
- Figure 3.27 The expanded plasmodesma contains a broad membrane-like structure which is continuous with e.r. membranes. x 160,000



and partly within the broad protoplast interconnexions which by now bear little resemblance to plasmodesmata. (Figs. 3.28 and 3.29). In Figure 3.30 a mitochondrion actually appears to be transferring from one protoplast to the adjacent one. Figure 3.31 shows a junction of three protoplasts (somewhat atypical as they do not form a longitudinal row). Broad connexions link the protoplasts with mitochondria and, as mentioned, stacks of e.r. apparently crossing them.

The changes described above take up to 18h to take place depending on the particular region involved. Plasmodesmatal expansion across transverse cell interfaces occurred considerably more rapidly than the much less frequent expansion across longitudinal ones. Any further changes beyond the condition shown in Figure 3.31 tended to be accompanied by some cytoplasmic breakdown. Figure 3.32 shows a fusion body in which the inter-protoplast junctions (arrowed) are rather obscured and there is almost complete isolation from adjacent laterally placed protoplasts. The fusion body however appears far from healthy, nuclear breakdown being extensive.

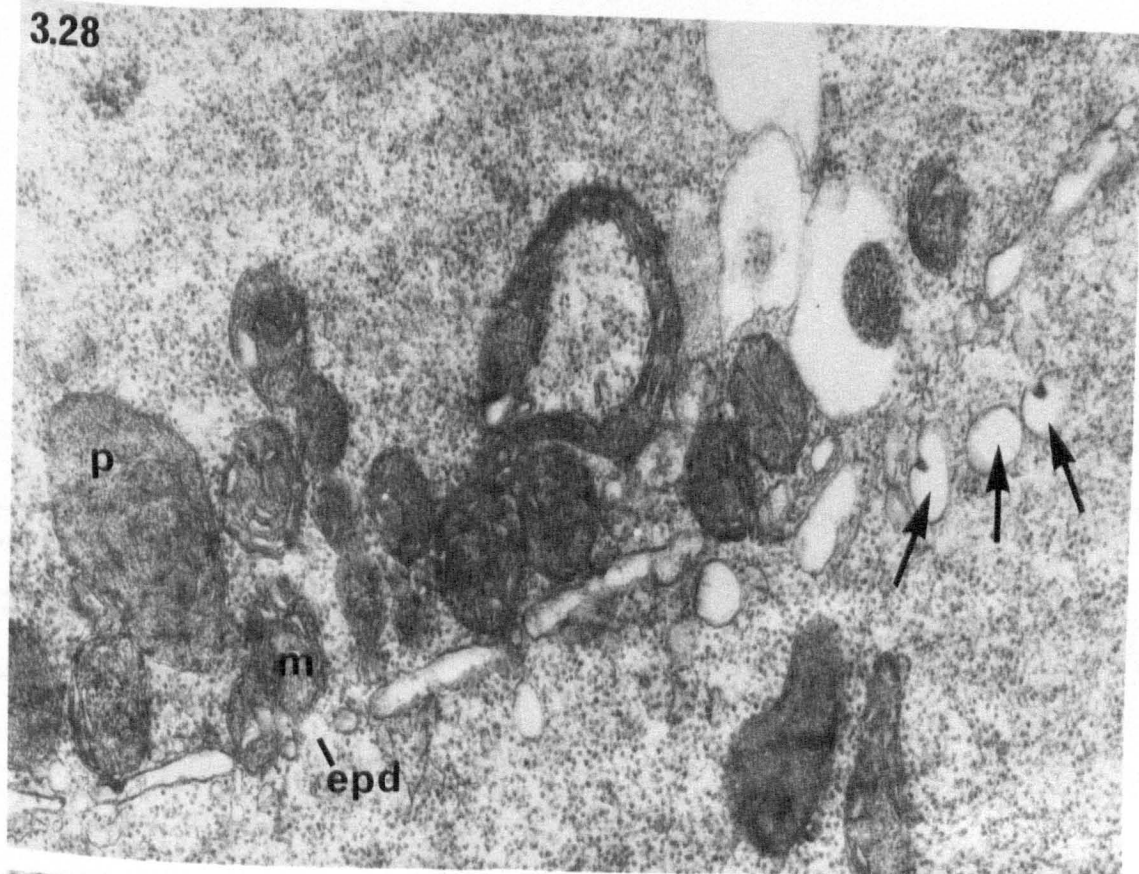
No further stages in rounding off were observed within the digested root tip, it being necessary for the fusion bodies to be released into a washing medium. Thus there appear to be limits to the transformations which can take place within the rigid structure of the root tip.

The fate of the plasmalemma which once separated the fusing protoplasts is not clear. In Figure 3.28 small vesicles (arrowed) break the line of expanding plasmodesmata and possibly represent displaced vesicles of plasmalemma. The electron dense molecule thorium dioxide ("Thorotrast", Fellows, Testagar, Detroit; British Supplier, Martindales, Bayswater), was added to the enzyme incubation medium in some preparations.

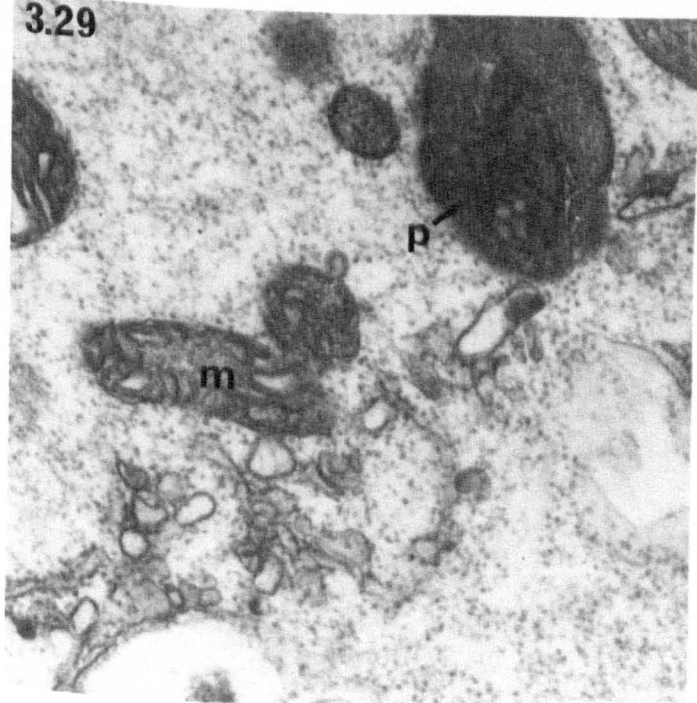
Enzyme digestion of the oat root tip
plasmodesmatal changes

- Figure 3.28 Plastids (p) and mitochondria (m) lie near to and partially within the expanded plasmodesmata. Several vesicles (arrowed) appear to be displaced from the protoplast interface. x 42,000
- Figure 3.29 As Figure 3.28. x 45,000
- Figure 3.30 A mitochondrion lies within an expanded plasmodesma (epd). x 44,000

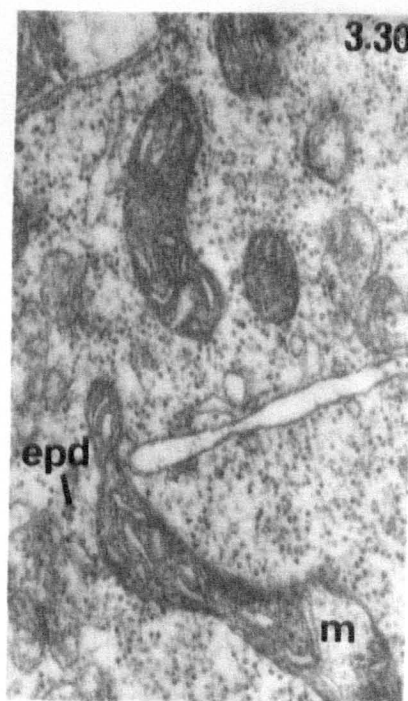
3.28



3.29



3.30



the same way as the other two, but the first two are not. The first two are not the same as the other two, but the first two are not the same as the other two.

the same way as the other two, but the first two are not. The first two are not the same as the other two, but the first two are not the same as the other two.

the same way as the other two, but the first two are not. The first two are not the same as the other two, but the first two are not the same as the other two.

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Enzyme digestion of the oat root tip

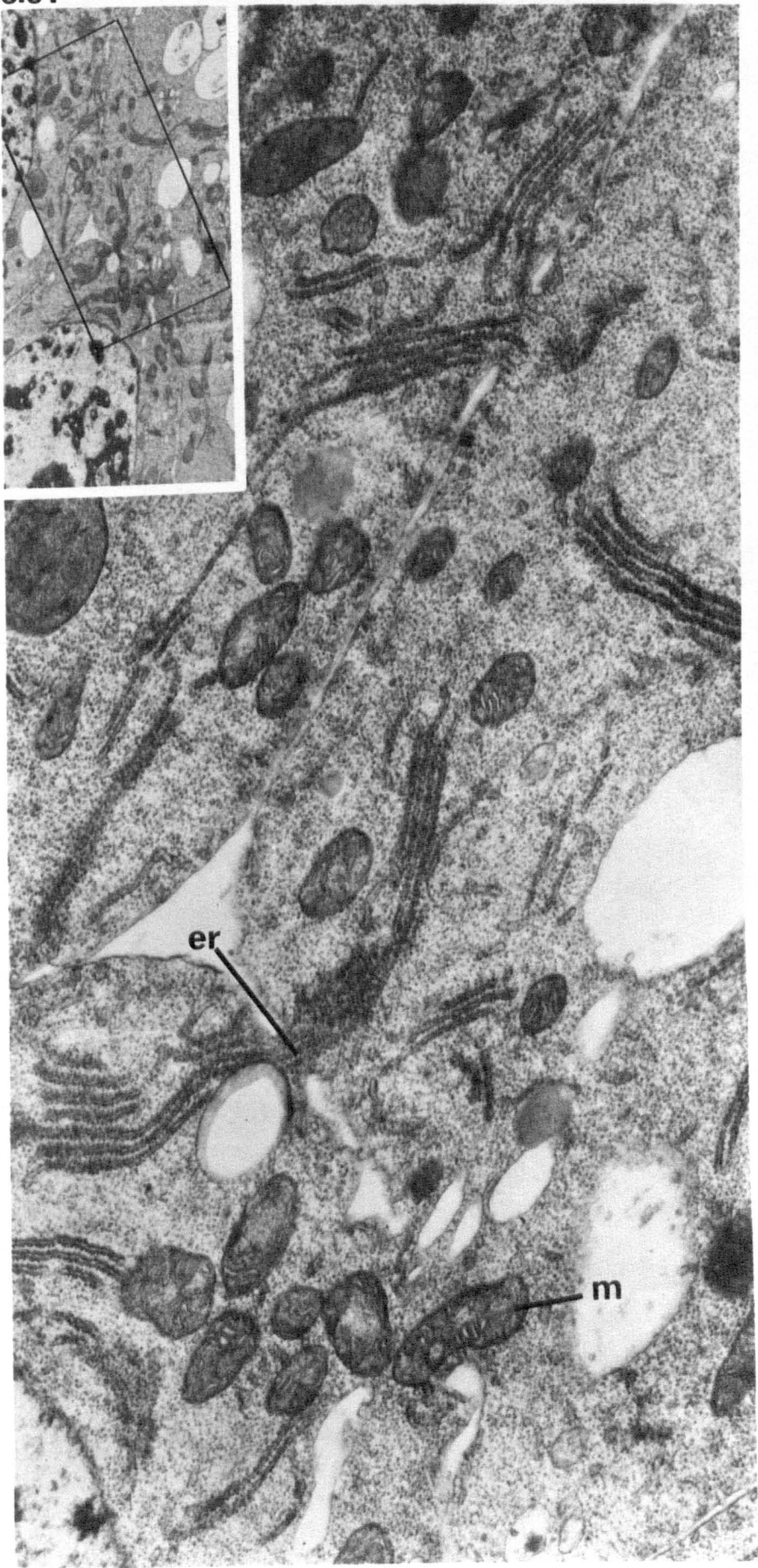
plasmodesmatal changes

Figure 3.31 Three protoplasts are linked by broad connexions.
They no longer resemble plasmodesmata. Mitochondria
and stacks of e.r. lie within the bridges.

x 35,000

inset x 7,000

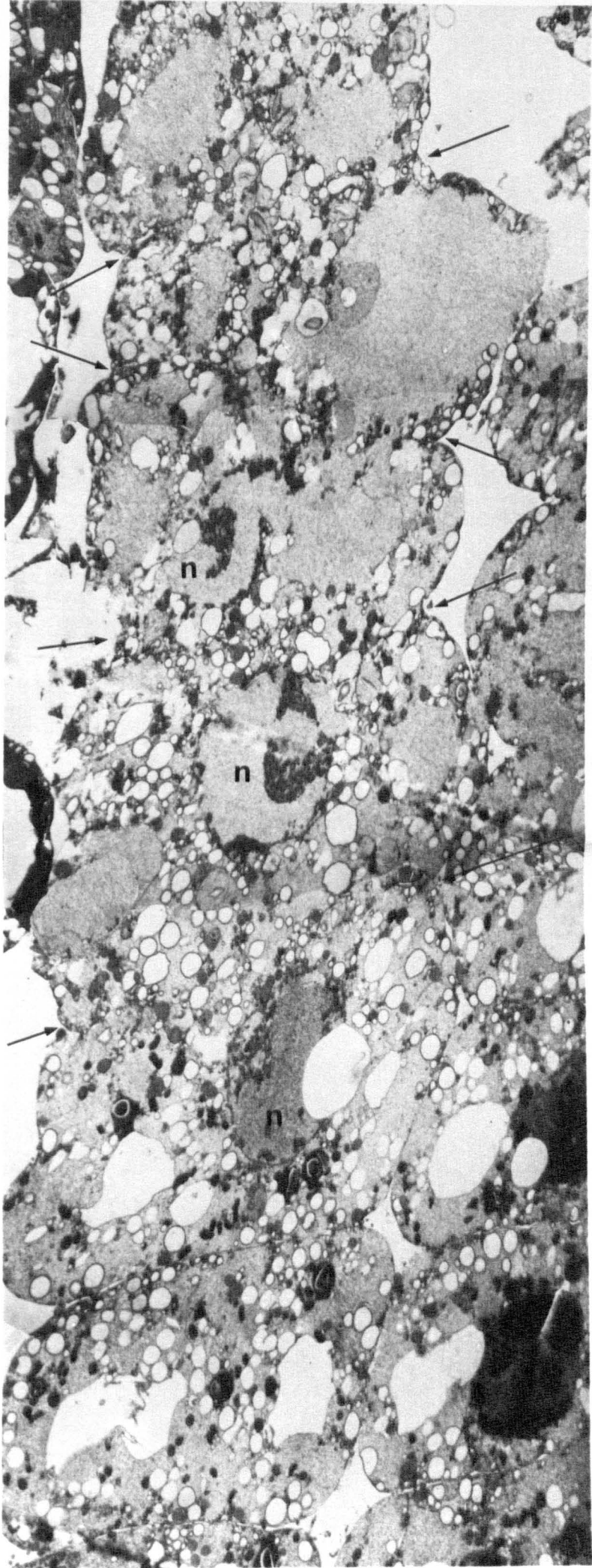
3.31



Enzyme digestion of the oat root tip
spontaneous fusion body formation

Figure 3.32 A linear fusion body has formed within the digested root tip. The inter-protoplast junctions are marked only by indentations in the outline of the fusion body (arrows). Nuclear breakdown is occurring. The fusion body is almost completely isolated from adjacent longitudinal rows of protoplasts.

x 4,500



It was hoped that this would enter the spaces created by plasmolysis and wall digestion and thus trace any vesicle formation by the plasmalemma. However the Thorotrast failed to penetrate the tissue mass.

It is possible to relate the changes in tissue organization occurring during enzyme treatment to a time course of events. Figure 3.33 shows diagrams of the root tip relating modifications during enzyme treatment to the original tissue structure. The stages shown, 6h and 24h, were selected since they most clearly demonstrated the progress of tissue digestion.

The root cap cells exhibited rapid and extreme plasmolysis but their walls remained completely undigested throughout. The cut end of the root tip was obviously mechanically damaged, making it susceptible to fixation damage. In this area wall digestion was moderate some wall remnants present even at 24h. The thick walled epidermis and sub-epidermal cells showed some wall digestion on the longitudinal walls adjoining the stele and plasmolysis was extreme.

The cells of the quiescent centre plasmolysed extremely throughout, with very few plasmodesmata remaining intact. There was no evidence of spontaneous fusion and by 24h only a little wall digestion had occurred. The adjacent stele cells (4 - 6 rows) behaved similarly but some of these, in the latter stages, digested more or less completely.

The most marked changes over the 6 - 24h period were in the stele itself. An area of cells plasmolysed into longitudinal lines, with intact transverse plasmodesmata and considerable wall digestion, covered the peripheral stele region and the stele cells near the cut end, at 6h.

By 24h the proximal half of the stele, peripheral and central, was

in this condition, whilst the more distal peripheral cells now exhibited plasmodesmatal expansion and fusion. The central stele cells, stretching from several cell rows above the quiescent centre to several rows from the cut end, plasmolysed in more or less the same pattern of longitudinal rows but less clearly, with little wall digestion occurring at 6h. By 24h this area had reduced to a few rows of cells 8 - 10 deep above the quiescent centre.

Spontaneous fusion was slow in progressing. At 6h only a small number of cells in the lower part of the stele exhibited much wall digestion and plasmodesmatal expansion. By 24h the most part of the lower half of the stele, excluding the few central cells and those immediately adjacent to the quiescent centre, was more or less completely digested, extensive spontaneous fusion occurring.


Reference to Table 3.1 shows that the regions of spontaneous fusion correspond somewhat with the stele regions having the highest ratio of plasmodesmata on transverse to longitudinal walls. It is noted that the epidermal, subepidermal and root cap regions, which also have moderate to high ratios but lack any sign of spontaneous fusion, also exhibit an extreme plasmolysis reaction and resistance to wall digestion.

Figure 3.33

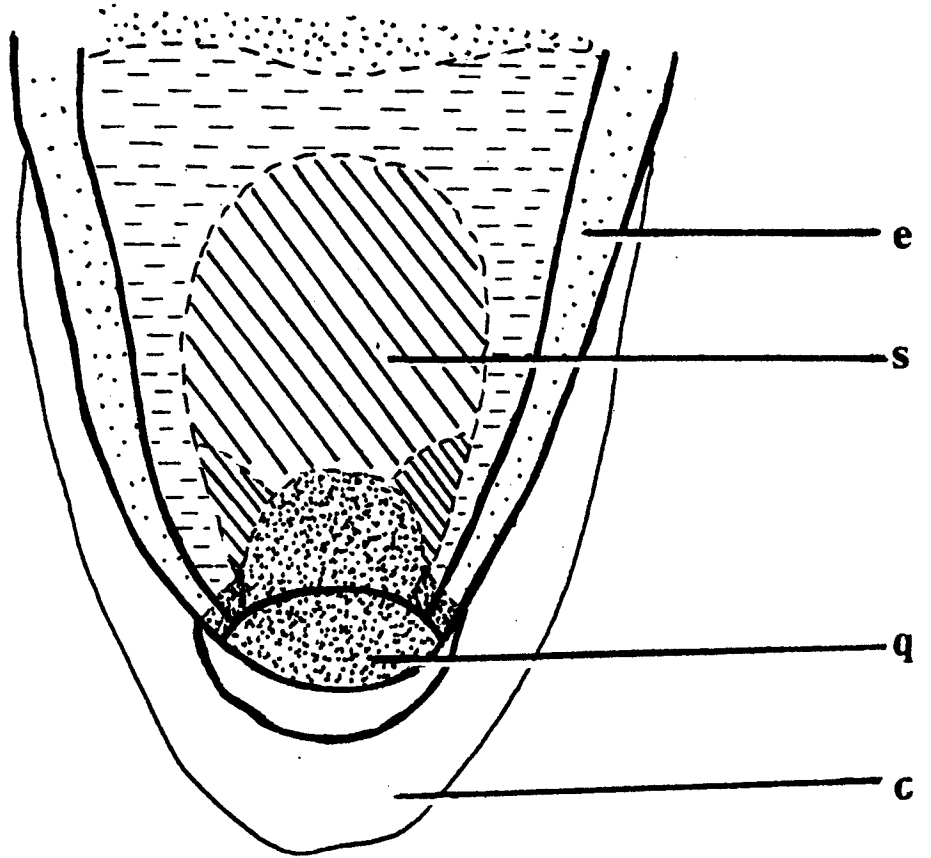
- a. Oat root tip after 6h enzyme treatment.
- b. Oat root tip after 24h enzyme treatment.

Regions of the root tip (as shown in Figure 3.1):-

- e = epidermis and sub-epidermis
- s = stele
- q = quiescent centre
- c = root cap

<u>Key</u>		Walls undigested, extreme plasmolysis, no fusion.
		Extensive wall digestion, mechanical fixation and enzyme damage, extreme plasmolysis, no fusion.
		Extreme plasmolysis, a little longitudinal wall digestion, no fusion.
		Considerable wall digestion, plasmolysis into longitudinal lines of cells, transverse plasmodesmata intact.
		Extreme plasmolysis, some plasmodesmata intact, little wall digestion, no fusion.
		Little wall digestion, plasmolysis into (mainly) longitudinal lines of cells, many plasmodesmata intact.
		Wall digestion complete, plasmodesmata expanded, extensive fusion.

a.



b.

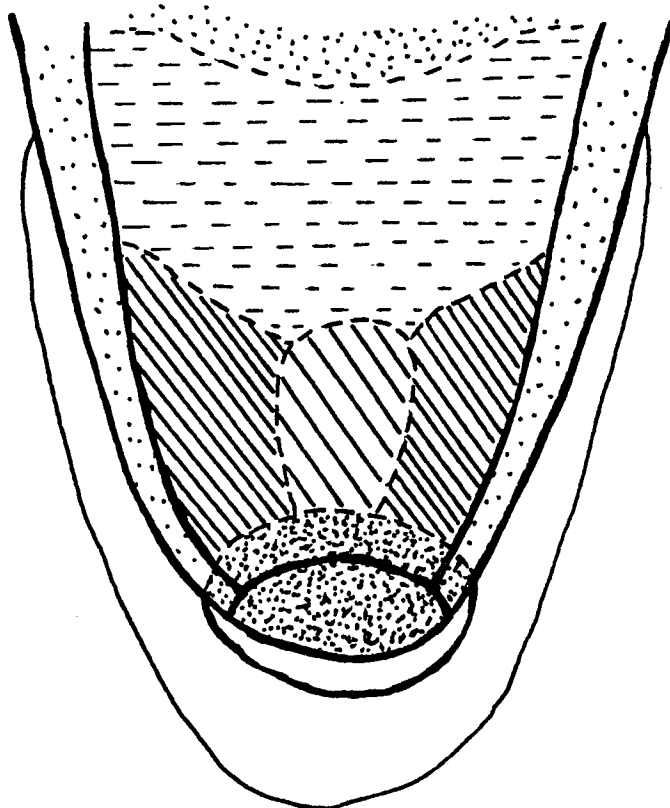


Figure 3.33

The progress of spontaneous fusion and associated tissue modifications in the enzyme treated oat root tip.
(see opposite)

Summary

(The oat root system)

The structure of the oat root tip is described, drawing on light and electron microscopic observations. Details of the distribution of plasmodesmata in the regions of the root tip are described. Generally the plasmodesmata are more numerous on walls connecting cells longitudinally related, i.e. transverse walls. Thus there is a greater continuity between cells in the longitudinal rows.

The pattern of enzyme digestion of the root tip is described, relating the response to enzyme treatment to the structure of the tissues. Regional variations in terms of tissue damage, the degree of tissue digestion and the location and form of spontaneous fusion bodies are described. A general linear form, reflecting the relationship of the cells in the parent tissue is observed.

A fine-structural study of plasmodesmatal changes during enzyme digestion indicates that whilst some plasmodesmata break, many resist the plasmolysis which accompanies enzyme treatment, and expand, drawing adjacent protoplasts together and facilitating organelle transfer.

3.2 THE MAIZE SEEDLING AND ONION SYSTEMS.

These systems were not studied in depth, but provided supporting evidence for a general pattern of enzyme digestion and protoplast release in tissues of the monocotyledonous type of construction.

3.2.1 The maize seedling.

The pattern of enzyme digestion of the maize seedling root tip follows closely that of the oat (3.1) producing single protoplasts, lines of cells and linear fusion bodies. (Figs. 3.34, 3.35 and 3.36 respectively.) As with the oat root tip, digestion is far from complete during the time of treatment. Any longer treatment was unsuccessful, being limited by tissue survival and enzyme activity factors. The coleoptile, however, digests much more completely and rapidly, producing again rows of cells and linear fusion bodies, the latter often assuming a more rounded shape. (Figs. 3.37, 3.38 and 3.39 respectively.)

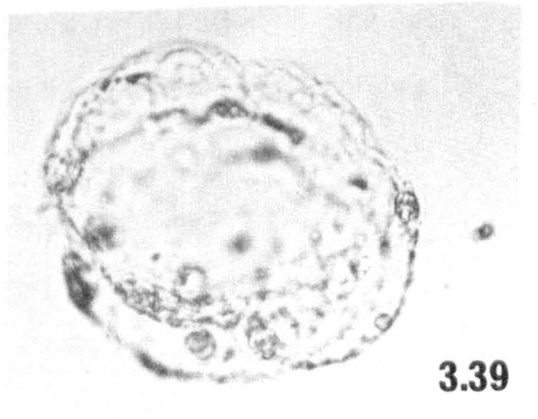
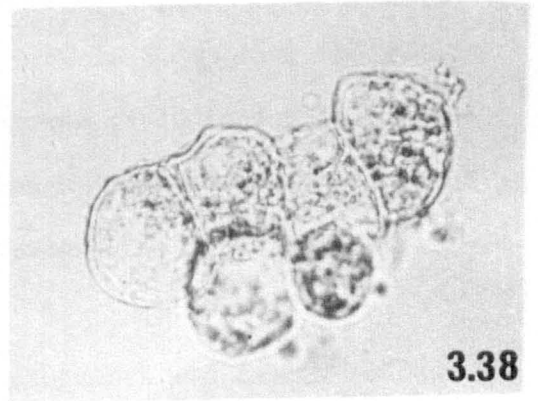
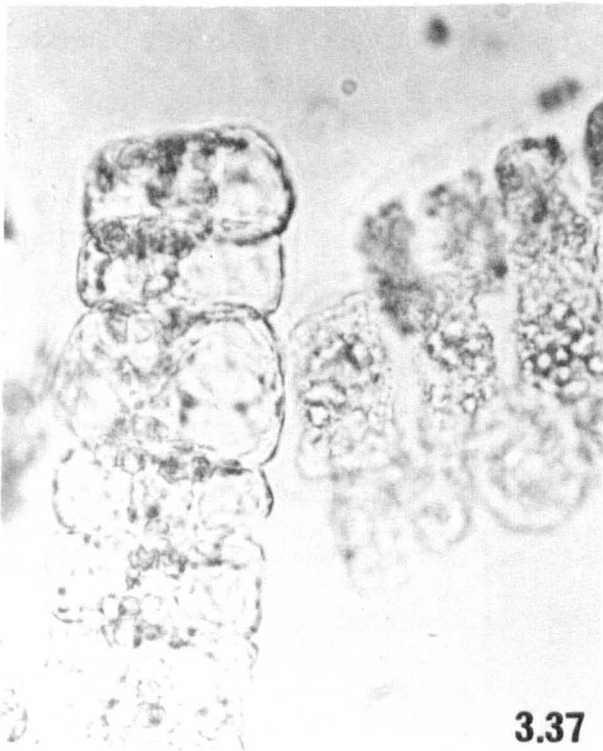
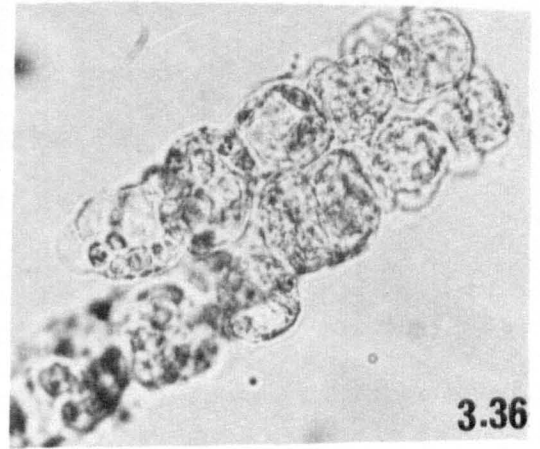
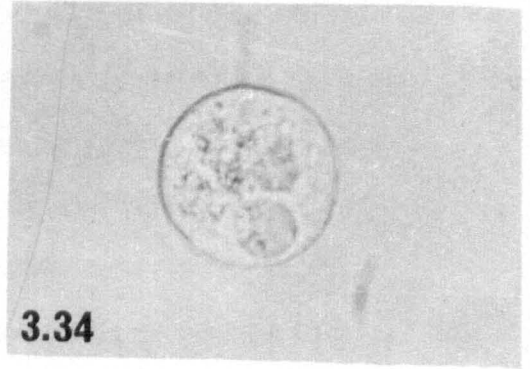
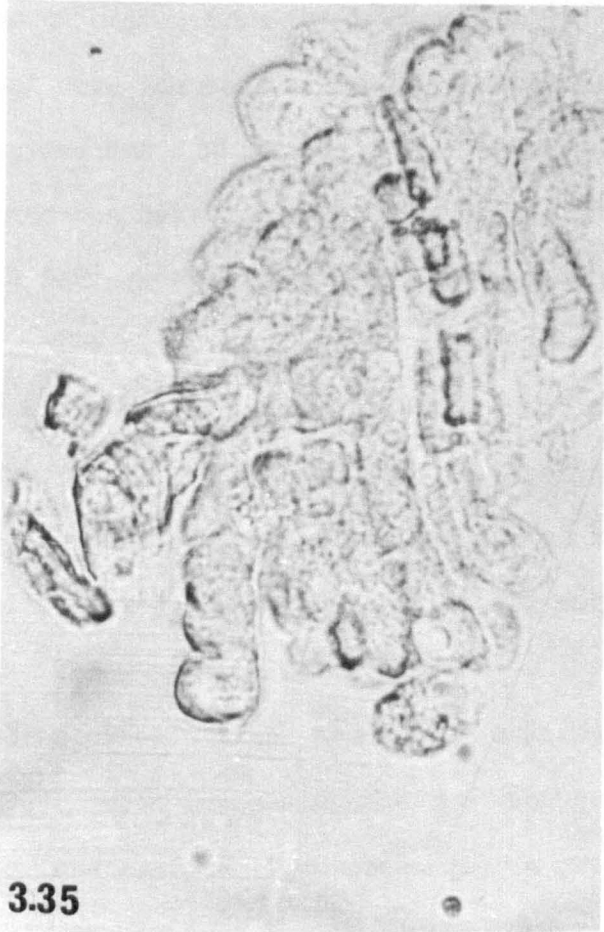
The first leaf differs from the root tip and coleoptile in that the majority of the cells are highly vacuolated. Many attached cells were produced and some fusion bodies (Figs. 3.40 and 3.41 respectively). The fusion bodies tended not to round off. Much of the tissue is left undigested. It is interesting to compare this with the enzyme digestion of the mature maize leaf. (Appendix 2.1).

3.2.2 The onion

The pattern of enzyme digestion of the root tip was again similar to the oat but markedly less extensive. This is probably due to the growing conditions and the characteristics of the tissue leading to a generally more robust construction.

Enzyme digestion of the maize
seedling root tip and coleoptile

Figure 3.34	One of the few single protoplasts isolated from the root tip.	x 400
Figure 3.35	The majority of the root tip tissues produced rows of cells as in this figure , or:	x 300
Figure 3.36	Linear fusion bodies were produced. Few rounded off.	x 400
Figure 3.37	The coleoptile digested more completely, producing rows of partially digested cells.	x 300
Figure 3.38	Linear fusion bodies were also produced.	x 200
Figure 3.39	Some fusion bodies rounded off.	x 350



A range of conditions were used in the isolation of protoplasts and fusion bodies from the leaf. (See 2.2.4). The older regions of the leaf were the most resistant to enzyme digestion, increase in cellulolytic enzymes and shaking the preparation during isolation increasing wall digestion only a little. The yield from this region was limited to a few dark green highly vacuolate single protoplasts (Fig. 3.42).

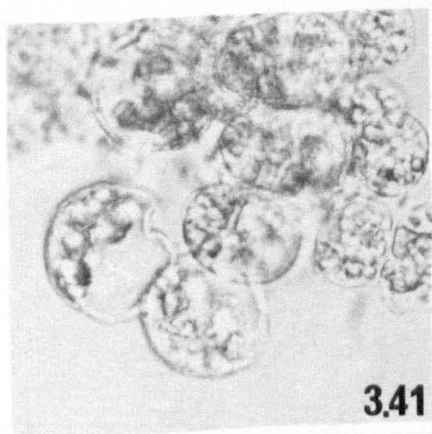
The pith, a slimy loosely structured tissue, produced many single highly vacuolate protoplasts and isolated vacuoles within a short time. (Fig. 3.43). The outer parts of the younger regions of the leaf gave good yields of protoplasts and fusion bodies. The response to increased cellulolytic enzyme and shaking was considerable.

The youngest region (from near the base of the bulb) produced masses of colourless, associated and fusing protoplasts. (Figs. 3.44 to 3.46). With increasing age the tissues showed an increase in vacuolation. The intermediate light green region produced more single protoplasts and fewer spontaneous fusion bodies, (Figs. 3.47 to 3.49) than the younger tissues. The oldest region to give a moderate yield tended to produce few if any spontaneous fusion bodies although lines of connected cells were observed. (Fig. 3.50). A general tendency to linearity was observed throughout.

The colourless highly cytoplasmic protoplasts and fusion bodies were stable in sucrose solutions as low as 2% (w/v), whereas the more vacuolate ones required at least 12-14% sucrose to prevent bursting. Thus the use of a particular osmoticum could be used to select a required fraction, the process being aided by the tendency of the more vacuolate protoplasts to float in high levels of sucrose.

Enzyme digestion of the maize seedling
first leaf and the onion leaf

- Figure 3.40 Maize: A row of attached cells. The walls are almost completely digested. x 550
- Figure 3.41 A little spontaneous fusion occurred during digestion of the leaf. The two lower protoplasts are possibly fusing. x 550
- Figure 3.42 Onion: Single dark green protoplasts produced from the oldest tissues of the leaf. x 400
- Figure 3.43 Highly vacuolated single protoplasts isolated from the pith. x 300
- Figure 3.44 Masses of attached cells/protoplasts formed from the youngest tissues. x 500
- Figure 3.45 Some spontaneous fusion occurred in these tissues forming linear fusion bodies. x 500
- Figure 5.46 A few of the fusion bodies rounded off. x 500
- Figures 5.47 and 5.49 Slightly older tissues were more vacuolate but still produced some spontaneous fusion bodies. Figure 3.47 x 450
Figure 3.49 x 500
- Figure 3.48 Some fusion bodies rounded off. x 450
- Figure 3.50 The oldest tissues to produce a moderate yield digested to form lines of attached cells and protoplasts. x 350



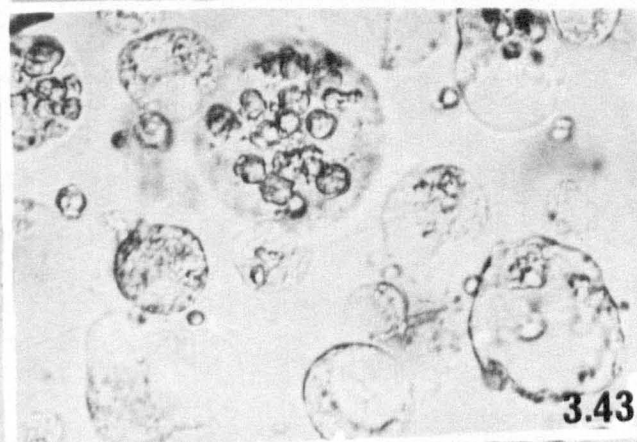
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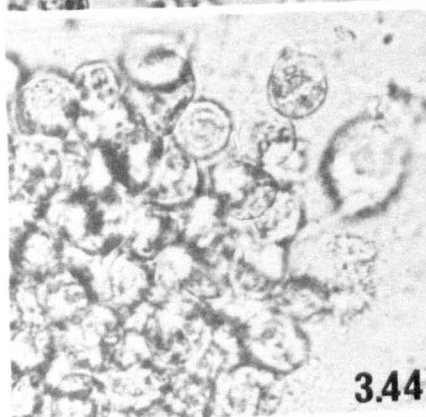
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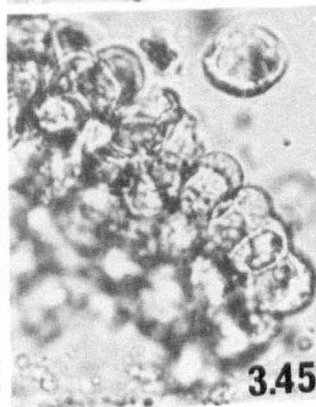
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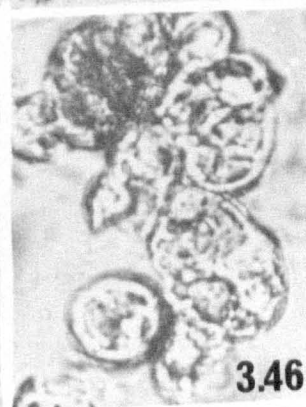
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3.44



3.45



3.46



3.47



3.49



3.48



3.50

Summary

(Monocotyledonous systems)

The enzyme digestion and protoplast and spontaneous fusion body release from the following tissues are described: onion root tip, onion leaf, maize root tip, maize seedling first leaf and coleoptile. Although there are certain differences within and between the various tissues, the results, in combination with those from the oat root system, suggest a common pattern of spontaneous fusion in monocotyledonous systems.

The cells in the undigested tissues are organized in longitudinal rows, relating to their origin. This linearity is reflected in the form of the fusion bodies, or where the process of spontaneous fusion is incomplete in the form of the digestion products - rows of attached and partially digested cells.

The degree of vacuolation of the tissue can limit the process. Generally spontaneous fusion occurs with a greater frequency in the less vacuolate tissues, and the fusion bodies which do form from the more vacuolate tissues are slow to round off, the vacuoles forming a physical restraint.

3.3 THE TOBACCO LEAF SYSTEM.

All the previously described tissues have common features in that they are from monocotyledonous plants with a general tendency, in the younger tissues, to a highly cytoplasmic condition. The organization of the cells in the tissues is markedly linear. As will be described, the tobacco leaf is in complete contrast to the above and the differences are reflected in the pattern of enzyme digestion, protoplast and spontaneous fusion body production.

3.3.1 The structure of the tobacco leaf.

Figure 3.51, a light micrograph of a thick section of plastic embedded leaf, gives a general idea of the tissue organization within the leaf. Upper and lower epidermes of thick walled colourless cells limit the leaf, enclosing the green photosynthetic mesophyll. This is itself divided into two layers, an upper closely packed palisade layer of elongate cells and a lower spongy mesophyll layer of loosely packed spherical or irregularly shaped cells. The latter layer contains the vascular tissues also.

The distribution of plasmodesmata was observed using thin sections in the electron microscope - (no detailed quantitative work was carried out). The epidermal cells had few plasmodesmatal connexions either mutual or to the mesophyll cells and they tended to be broken by plasmolysis. Over the mesophyll cell walls there tended to be a fairly even distribution of plasmodesmata but, importantly, the areas of connecting wall varied in different regions. Adjacent palisade layer cells had considerable areas of contact, some five times greater than adjacent palisade and spongy mesophyll or pairs of spongy mesophyll cells. Accordingly, the plasmodesmatal connexions were proportionately

more numerous. Plasmodesmata rarely occurred singly and frequently their association was marked by a central wall complex. (Robards 1971), (Fig. 3.60, arrow).

3.3.2 Enzyme digestion and protoplast release: light microscopic observations.

A quantitative comparison of the various incubation media used in the isolation of tobacco leaf protoplasts is given in section 3.5.3. Such considerations are omitted here; digestion is assumed to be carried out to completion over a period of approximately 18h.

The lower epidermis removed, the spongy mesophyll is exposed to the enzyme incubation medium. The loose attachment of the epidermis led to little structural damage resulting from this operation and therefore most of the spongy mesophyll was available for protoplast production.

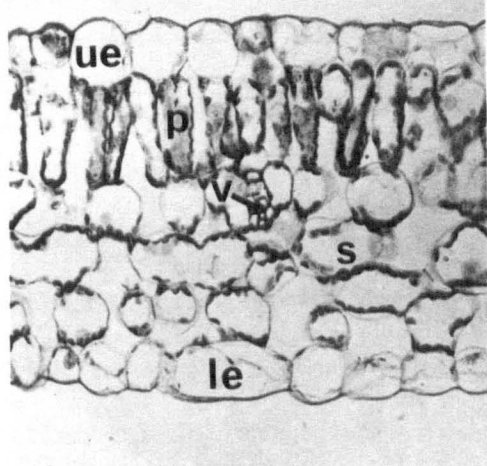
Within 4 - 6h, complete spongy mesophyll digestion had occurred, leaving behind the upper epidermis, palisade mesophyll and vascular tissues. (Fig. 3.52). The digestion produced a mixture of single protoplasts and irregularly shaped spontaneous fusion bodies. (Fig. 3.53). Further digestion involved the now exposed, quasi-monolayer of palisade mesophyll (Fig. 3.52), producing single protoplasts plus large flat fusion bodies in the form of a two-dimensional array, one protoplast deep. (Fig. 3.54). Frequently the edges of the fusion body showed signs of damage (Fig. 3.54 arrows). When the isolate was observed at a later stage in the enzyme digestion, the fusion bodies assumed a more compact, rounded form. (Figs. 3.55 and 3.56).

The final stages of enzyme digestion, released protoplasts from the upper epidermis. (Appendix 2.2).

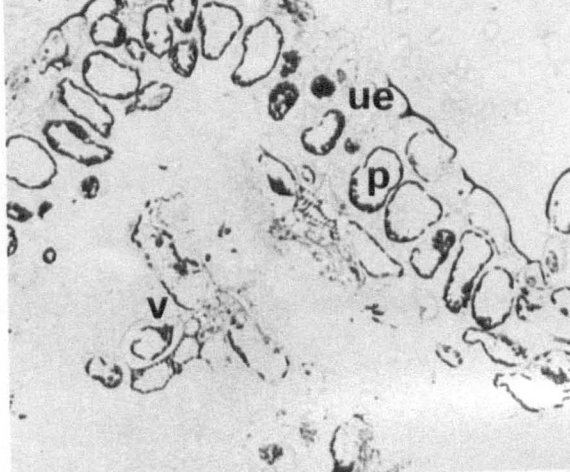
Enzyme digestion of the tobacco leaf

- Figure 3.51 A section through an untreated tobacco leaf. The upper epidermis (ue) and lower epidermis (le) limit the leaf tissues. The pallisade mesophyll (p) lies beneath the upper epidermis and the spongy mesophyll (s) lies between the pallisade layer and the lower epidermis. Vascular tissues (v) run through the spongy mesophyll. x 200
- Figure 3.52 A section through a partially digested tobacco leaf. The upper epidermis and pallisade layer remain undigested, the latter, somewhat rounded compared with the above figure. The vascular tissues are loosely attached. x 200
- Figure 3.53 Irregularly shaped spontaneous fusion bodies isolated from the spongy mesophyll. x 160
- Figure 3.54 A large flat spontaneous fusion body isolated from the pallisade layer, with some single protoplasts. Arrows indicate points of damage. x 160
- Figures 3.55 and 3.56 Large fusion bodies in stages of rounding off, with some smaller fusion bodies and single protoplasts. x 140

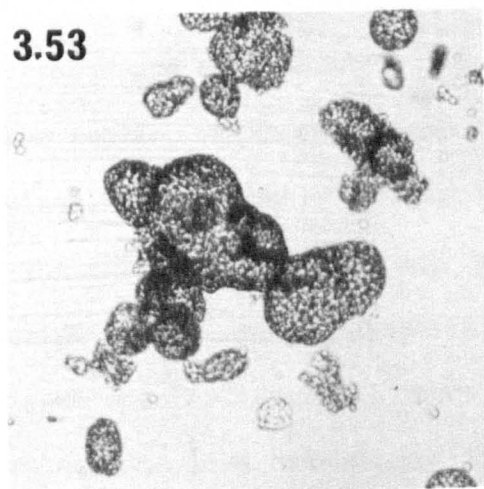
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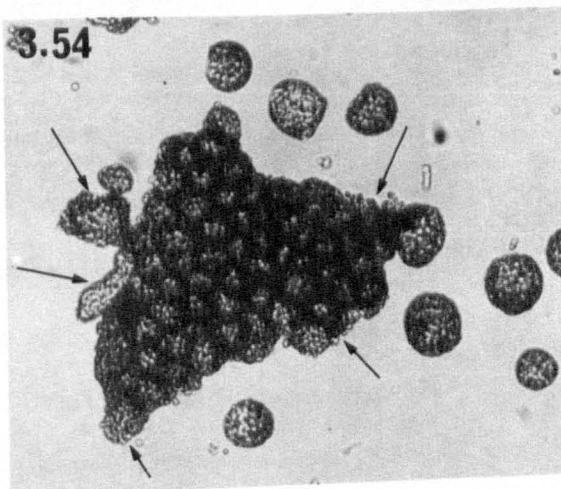
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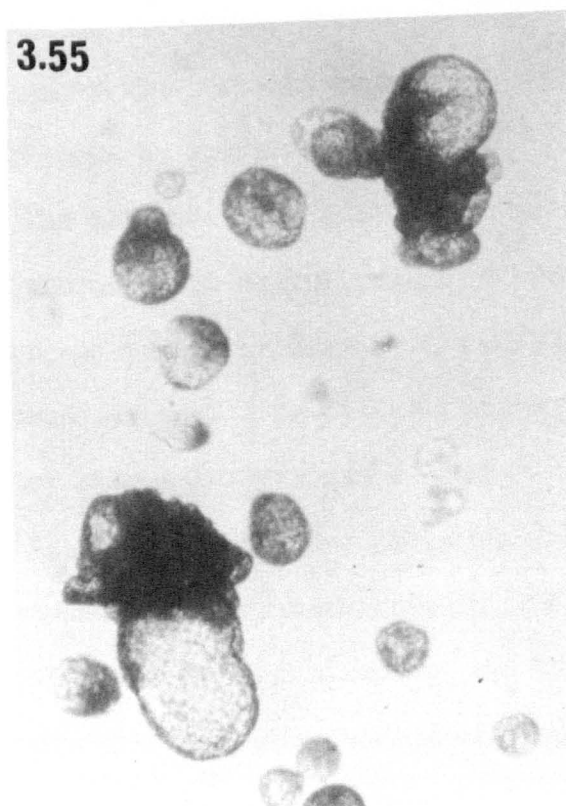
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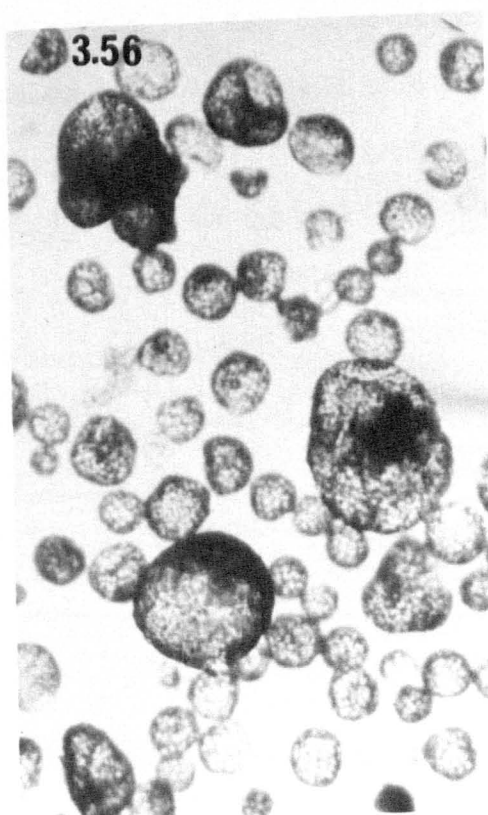
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3.55



3.56



3.3.3 Enzyme digestion and protoplast release: electron microscope observations.

As in the oat root, the effects of plasmolysis frequently preceded and always accompanied wall digestion. Figure 3.57 shows three adjacent spongy mesophyll cells with many plasmodesmatal connexions. Some plasmolysis has occurred, withdrawing the plasmalemma and possibly breaking some of the plasmodesmata. Figure 3.58 shows a similar situation but following some wall digestion. Here cytoplasmic fragments lie between the protoplast and the partially digested wall. These two micrographs are of moderately thin sections unlike Figure 3.59, a thicker section which shows more clearly the formation of strands of cytoplasm, some probably joining the protoplasts to the cell wall.

The extreme stretching and frequent breakage of plasmodesmata occurring in the spongy mesophyll is not, however, repeated in the palisade mesophyll tissue. Here plasmolytic withdrawal of the plasmalemma is less extreme and the plasmodesmata, often grouped and complex in structure, resist breakage. (Fig. 3.60). This is so despite the longer time between exposure to the plasmolyticum and wall digestion experienced by the palisade layer.

The plasmodesmata remaining intact showed some increased clarity of structure when wall digestion occurred. (Figs. 3.61 and 3.62). As in the oat root, the desmotubule within the plasmodesma became visible. An association of e.r. with the plasmodesmata was again evident. No further stages in spontaneous fusion were observed within the leaf. That is, protoplasts were released either singly or with narrow plasmodesmatal connexions between them. (Figs. 3.63, 3.64 and 3.65). Thus the looseness of structure and rapidity of wall digestion in the leaf deprive the fusion bodies of support at this critical stage. By

Enzyme digestion of the tobacco leaf

Figure 3.57 A region of plasmolysed spongy mesophyll tissue. Many plasmodesmata traverse the cell walls. The thin sectioning gives the incorrect impression of breaks in the plasmodesmata. Most remain connected to the plasmolysed protoplasts. Note the complex plasmodesmatal structure.

x 12,000

Figure 3.58 Some wall digestion has occurred. The protoplast, left, is highly plasmolysed. Apparent cytoplasmic fragments lie between it and the wall remnants (arrows) x 14,000

Figure 3.59 A thicker section than the above figure. It shows that many of the cytoplasmic fragments are in fact strands.

x 3,000

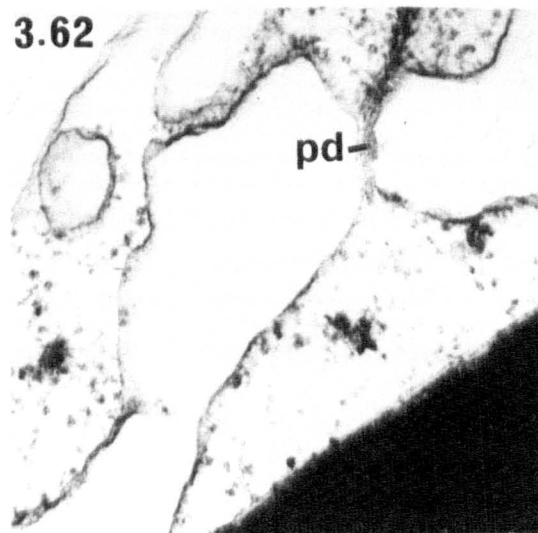
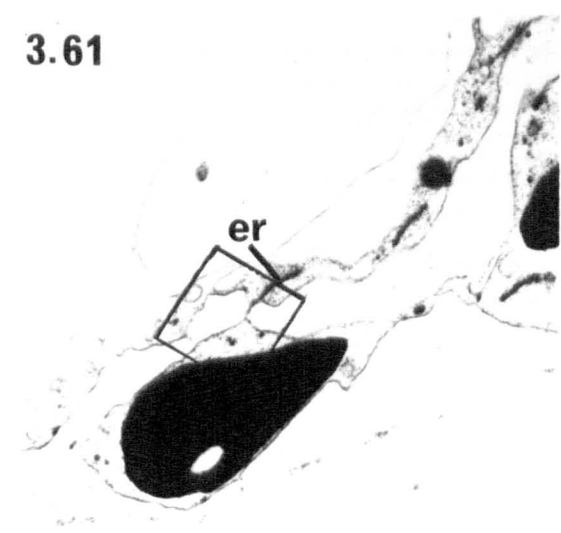
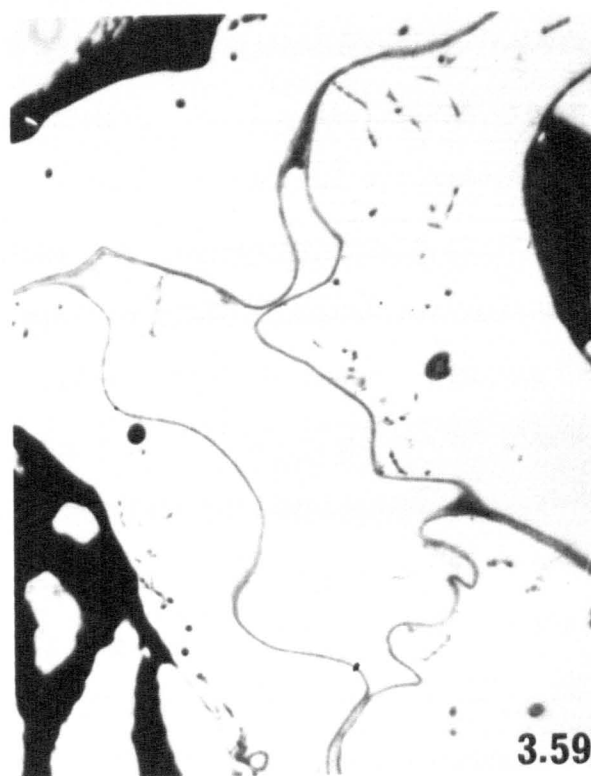
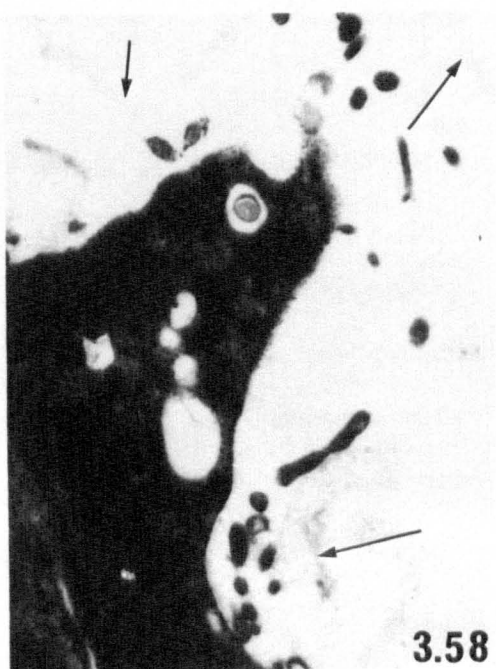
Figure 3.60 Two adjacent palisade layer cells. In this region plasmodesmata frequently resist plasmolytic breakage. The central wall complex of one of the plasmodesmata is arrowed.

x 12,000

Figures 3.61 and 3.62 Wall digestion is almost complete. Two plasmodesmata remain linking adjacent protoplasts. An e.r. membrane can be seen attached to the end of one of the plasmodesmata.

Figure 3.61 x 14,000

Figure 3.62 x 75,000



disturbing the tissues at this time the level of spontaneous fusion can be greatly reduced.

The structure of the plasmodesmata joining protoplasts frequently mirrors that seen in the undigested tissue, except in that the central wall complex appears more rounded. (Figs. 3.64 and 3.65 arrows, c.f. Fig. 3.60). Figure 3.65 shows two protoplasts joined by plasmodesmata and broader connexions. Other protoplasts are joined exclusively by these, no plasmodesmata being apparent. (Figs. 3.66 to 3.71). The two protoplasts in Figures 3.66 and 3.67 are joined by two broad connexions, one of which is apparently (in the section) a complex of four smaller connexions. Similar formations are seen in Figures 3.68 to 3.70).

Little evidence of a plasmodesmatal origin is apparent although in Figure 3.70 the relationship of the e.r. to one of the connexions (arrow) is reminiscent of plasmodesmata/e.r. relationships. Additionally the interconnexions shown in Figure 3.71 have a grouping suggestive of an origin from fields or groups of plasmodesmata.

The fate of the plasmalemma between fusing protoplasts is not clear. As mentioned, the connexions between fusing protoplasts are subdivided, being separated by what appear in thin section to be vesicles. (Fig. 3.70, double arrows), but probably represent ramifying extracellular space, at least in the early stages of fusion. To determine whether this space became enclosed by plasmalemma, forming internal vesicles, Thorotrast was included in the enzyme incubation medium in some preparations. The results were unexpected, it being impossible to draw any conclusions regarding the problem outlined above.

To explain: A tissue fixed during plasmolysis, with no wall digesting enzymes present, shows deep invaginations, and undulations in its outline, suggesting a folding of the protoplast to accommodate its

Tobacco leaf system

plasmodesmatal changes following enzyme digestion

(Stars on low power figures indicate regions shown at higher power in following figures)

Figures 3.63 and 3.64 Two protoplasts are joined by narrow plasmodesmatal connexions. The central wall complex of one of the plasmodesmata is arrowed. Figure 3.63 x 4,500

Figure 3.64 x 30,000

Figure 3.65 As above. Again the central wall complex is arrowed.

It is of a more rounded shape than in the undigested tissue. x 12,000

Figures 3.66 and 3.67 Two protoplasts are joined by broader connexions.

That to the right appears to be made up of 4 (in the section) smaller connexions. Figure 3.66 x 1,500

Figure 3.67 x 10,000

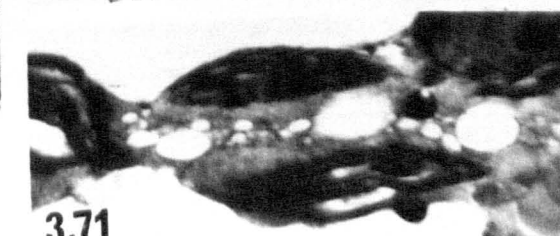
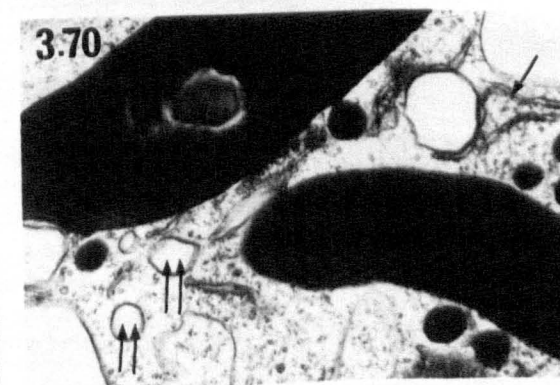
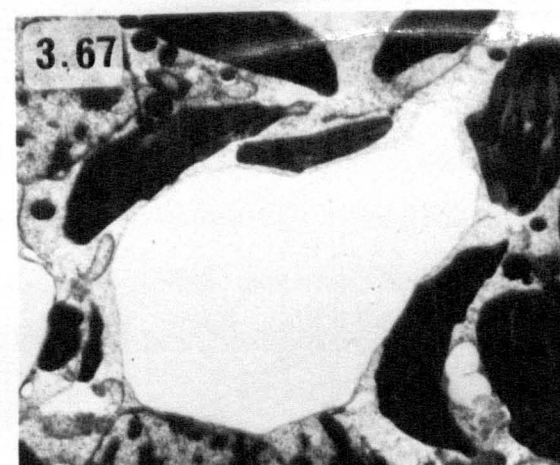
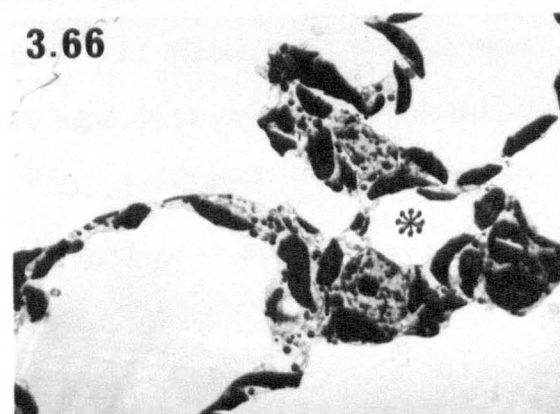
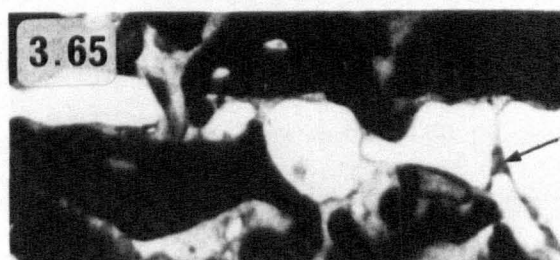
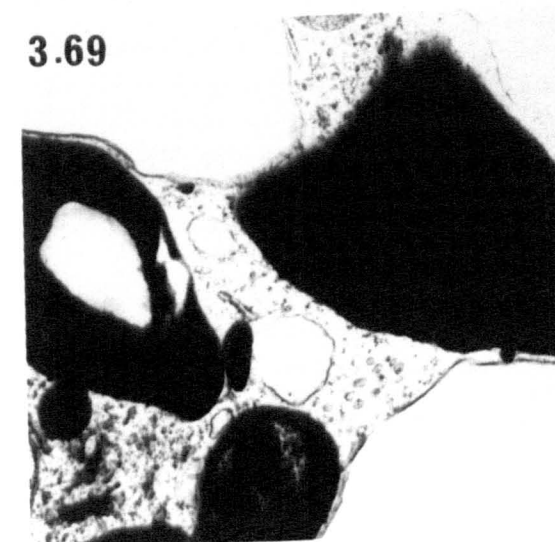
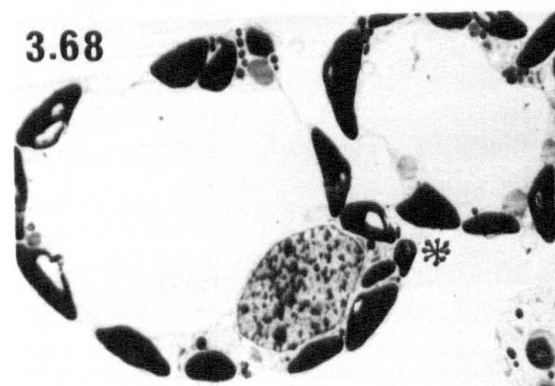
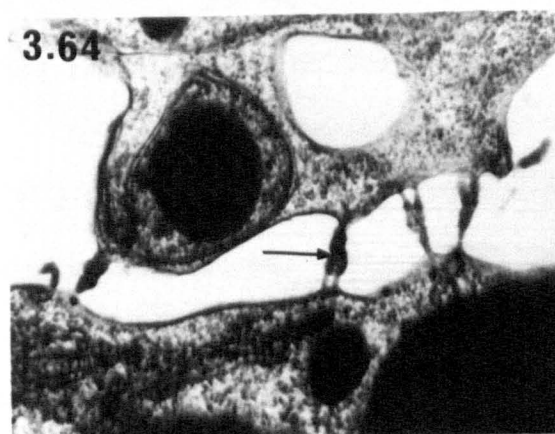
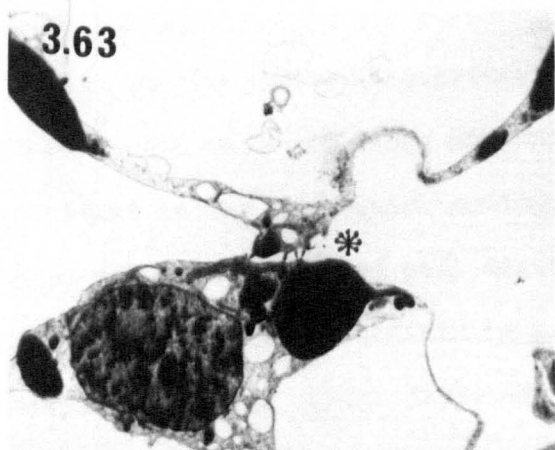
Figures 3.68 and 3.69 A similar stage to the above.

Figure 3.68 x 3,000

Figure 3.69 x 16,000

Figure 3.70 A very broad interconnecting region is apparently subdivided by vesicles (double arrows). The location of e.r. membranes suggests a plasmodesmatal origin for the subunits of the complex connexion. (membranes are indicated by a single arrow) x 25,000

Figure 3.71 The many protoplast interconnexions are grouped, resembling fields of plasmodesmata. x 9,500



decrease in size upon plasmolysis. (Fig. 3.72). The same process is observed with wall digesting enzymes present. With the inclusion of Thorotrast in the incubation medium the following are seen. In the early stages of plasmolysis and wall digestion, "bubbles" enclosed by a thin layer of cytoplasm and containing particles of Thorotrast and fibrous material are seen. (Fig. 3.73). By the time that protoplast isolation is complete, the Thorotrast is enclosed in vesicles which are integrated into the structure of the protoplast. (Figs. 3.74 and 3.75).

By preceding enzyme treatment with plasmolysis for 2 - 4h in e.g. 25% w/v sucrose solution, the amount of Thorotrast incorporation could be greatly reduced, (Fig. 3.76) although considerable vesiculation still takes place. At the same time, spontaneous fusion was almost totally absent. Thus this technique is unsuitable on two counts for identifying the plasmalemmar vesicles described earlier: firstly considerable uptake occurs during plasmolysis and secondly the treatment used to prevent this also prevents spontaneous fusion from occurring.

3.3.4 Post incubation changes in spontaneous fusion bodies.

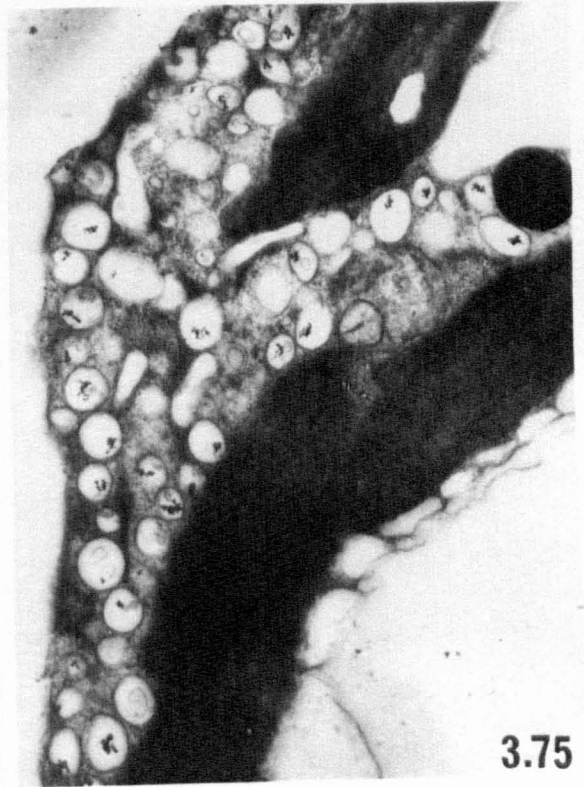
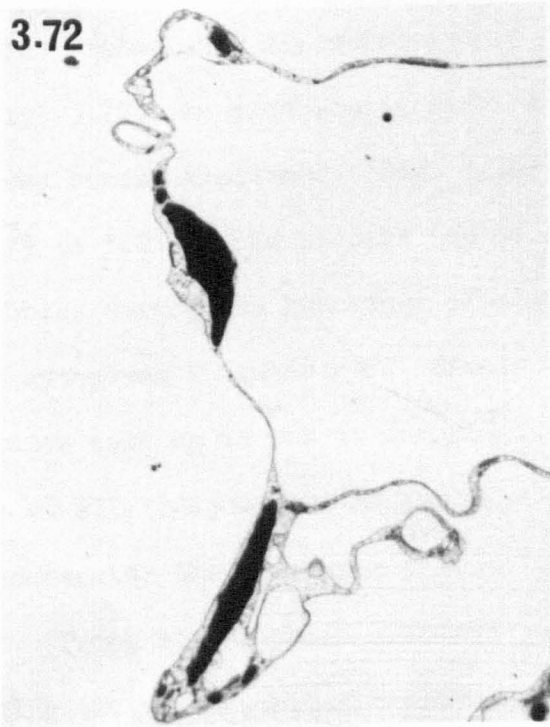
As mentioned in 3.3.2, the shape of spontaneous fusion bodies depends upon when they are observed since they tend to become more rounded with time. With small fusion bodies, rounding off is probably a fairly simple process involving a transformation from a lobed to a rounded shape, with corresponding internal reorganization. However for larger fusion bodies, particularly those from the palisade mesophyll, rounding-off would appear to be rather more complex. There are observed two modes of rounding off in large fusion bodies, depending on the treatment of the preparation.

Tobacco leaf system.

vesiculation and uptake during plasmolysis.

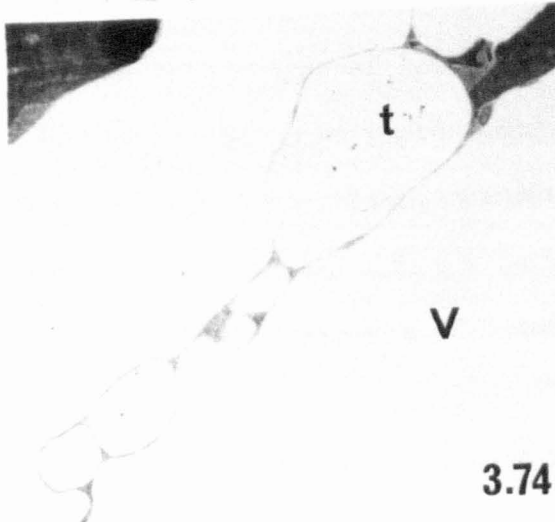
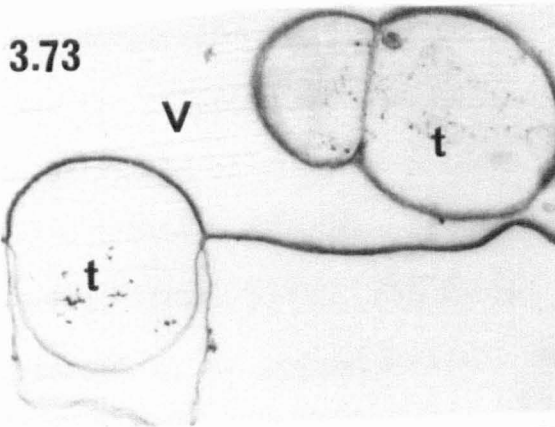
- Figure 3.72 A protoplast undergoing plasmolysis. Invagination of the plasmalemma appears to be taking place. x 6,000
- Figure 3.73 Thorotrast (t) , included in the enzyme incubation mixture, is taken up into 'bubbles' which are enclosed by a thin layer of cytoplasm. (V=vacuole) x 24,000
- Figure 3.74 As above, but here the newly formed vesicles are incorporated into the cytoplasm. x 18,000
- Figure 3.75 At a later stage, the thorotrast is seen within vesicles in the main cytoplasmic mass. x 21,000
- Figure 3.76 A protoplast plasmolysed before exposure to the thorotrast, shows considerable vesiculation but little uptake of the thorotrast.(arrowed) x 5,500

3.72

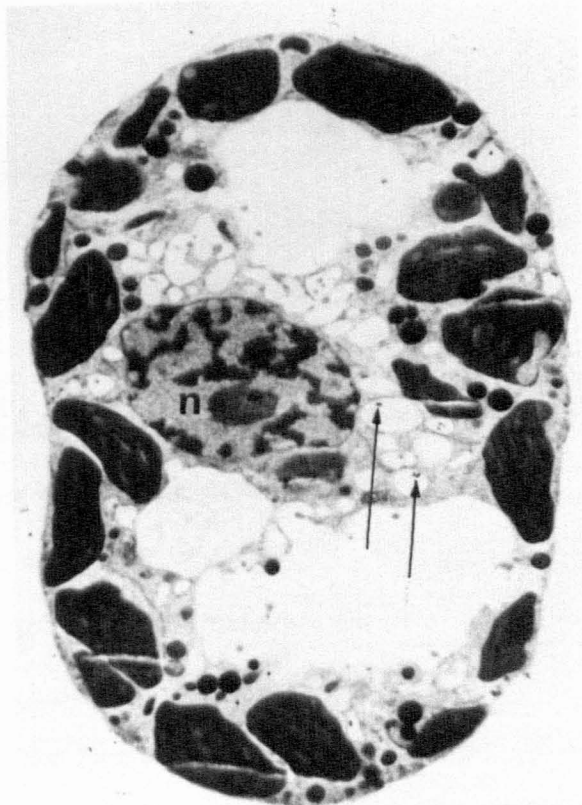


3.75

3.73



3.74



3.76

If the preparation is disturbed at the stage when the pallisade layer protoplasts are sufficiently fused to hold together in a layer, (Fig. 3.77) the protoplasts interfaces are clearly seen. With time these become flattened, (Fig. 3.78) and eventually disappear. (Figs. 3.79 to 3.81). The process can be compared with the merging of bubbles during the breakdown of a gas liquid emulsion. No mass movement of cytoplasm is envisaged. Simply a fusing of vacuoles. Such fusion bodies take up to 36h to completely round off although some fail to do so at all. Adjustment of the plasmolyticum to a lower concentration accelerates the process a little.

Preparations which progress to complete tissue digestion before being disturbed exhibit a different mode of rounding off. Figure 3.55 shows compact fusion bodies with a polarity of cytoplasm and vacuole. Later the polarity is less obvious but there is still a local concentration of cytoplasm. (Fig. 3.56). In the simplest of these fusion bodies one large vacuole is observed but in others there are several. (Fig. 3.82a). The fusion body in Figure 3.82b shows some degree of damage in one region (arrow), on contrast to the several damaged points in Figure 3.54 (arrows).

This latter type of fusion body is more susceptible to osmotic manipulation, the most rapid rounding off being produced by using a high osmoticum during isolation, changing to a low one for the washing medium. (e.g. 16 and 10% w/v sorbitol respectively). It is interesting to note that the inclusion of Cytochalasin B (Serva, Heidelberg) at levels of 5 μ g/ml to 100 μ g/ml during enzyme treatment slightly increases the yield of large flat fusion bodies, but totally prevents their rounding off. (See section 3.5.3b)

To investigate both the general and fine structure of fusion bodies

Tobacco leaf system

Spontaneous fusion body shape changes

Figure 3.77 A fusion body isolated at a very early stage.

The individual protoplasts are clear identified.

x 150

Figure 3.78 Some merging of adjacent protoplasts is occurring.

The interfaces are flattened.

x 300

Figure 3.79 Many of the protoplast interfaces have disappeared.

x 150

Figures 3.80 and 3.81 Two large , irregularly shaped fusion

bodies. Few protoplast interfaces can be identified.

The vacuoles appear to have fused.

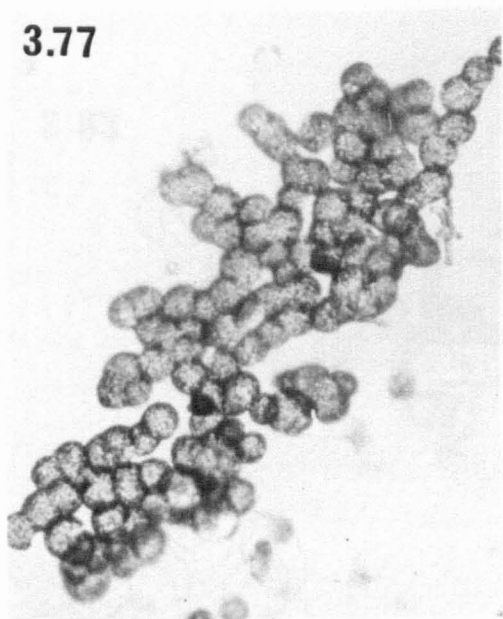
Figure 3.80 x 100

Figure 3.81 x 150

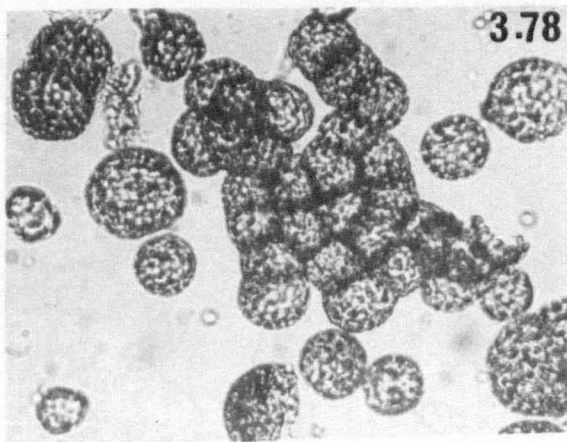
Figures 3.82a and b Two large polarised fusion bodies. Each body contains one or more vacuolated regions and a highly cytoplasmic region. There is frequently an area of damage in the latter region (arrow).

x 275

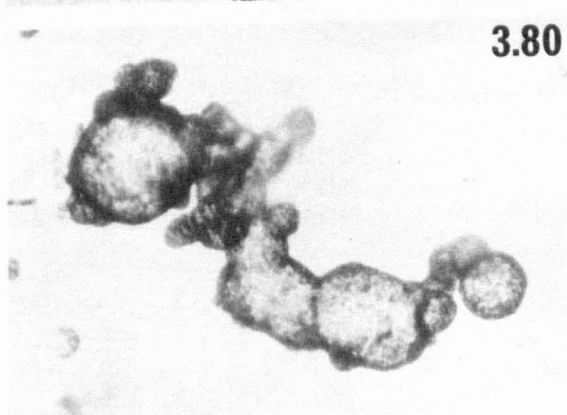
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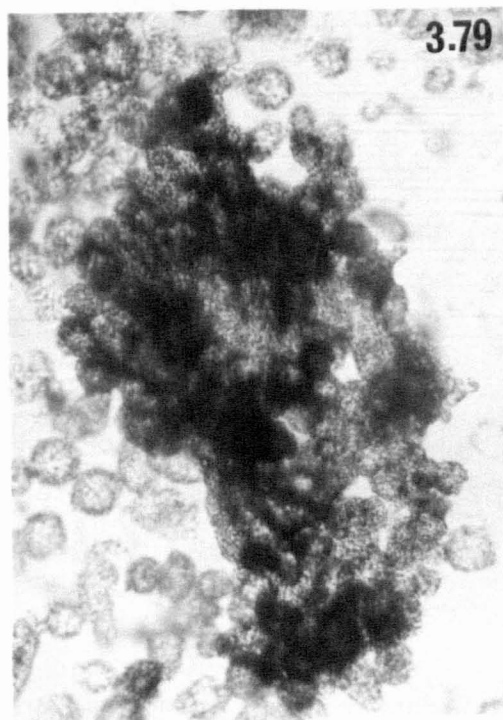
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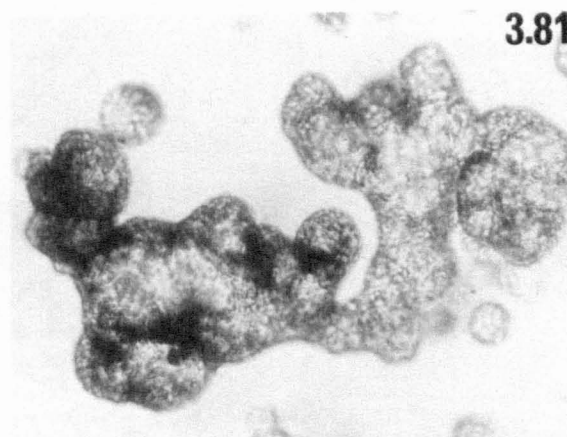
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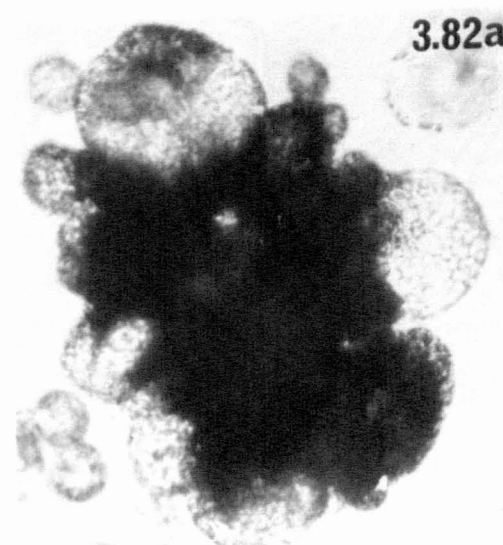
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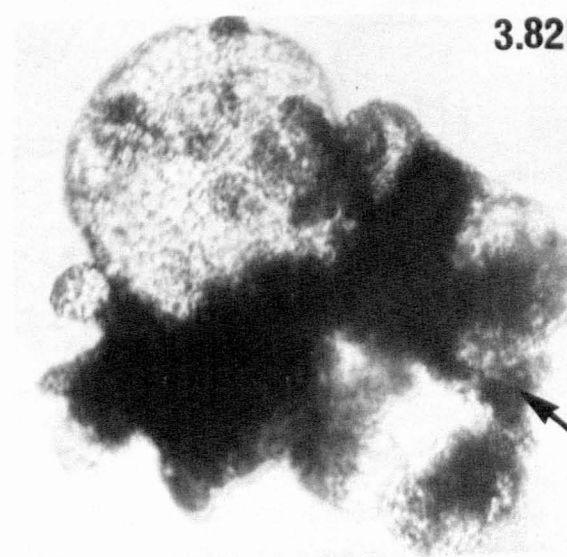
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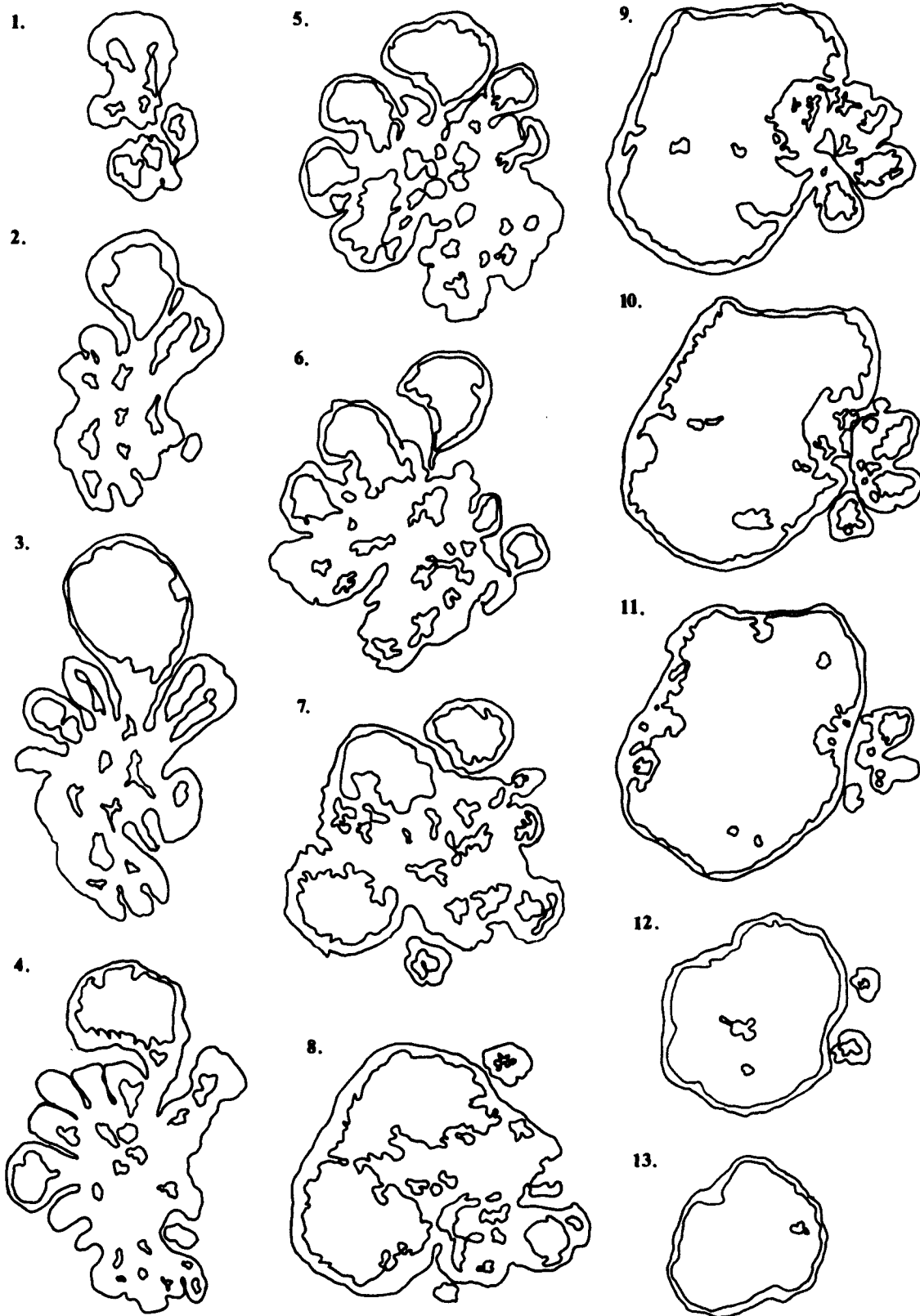
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3.82b



3.83

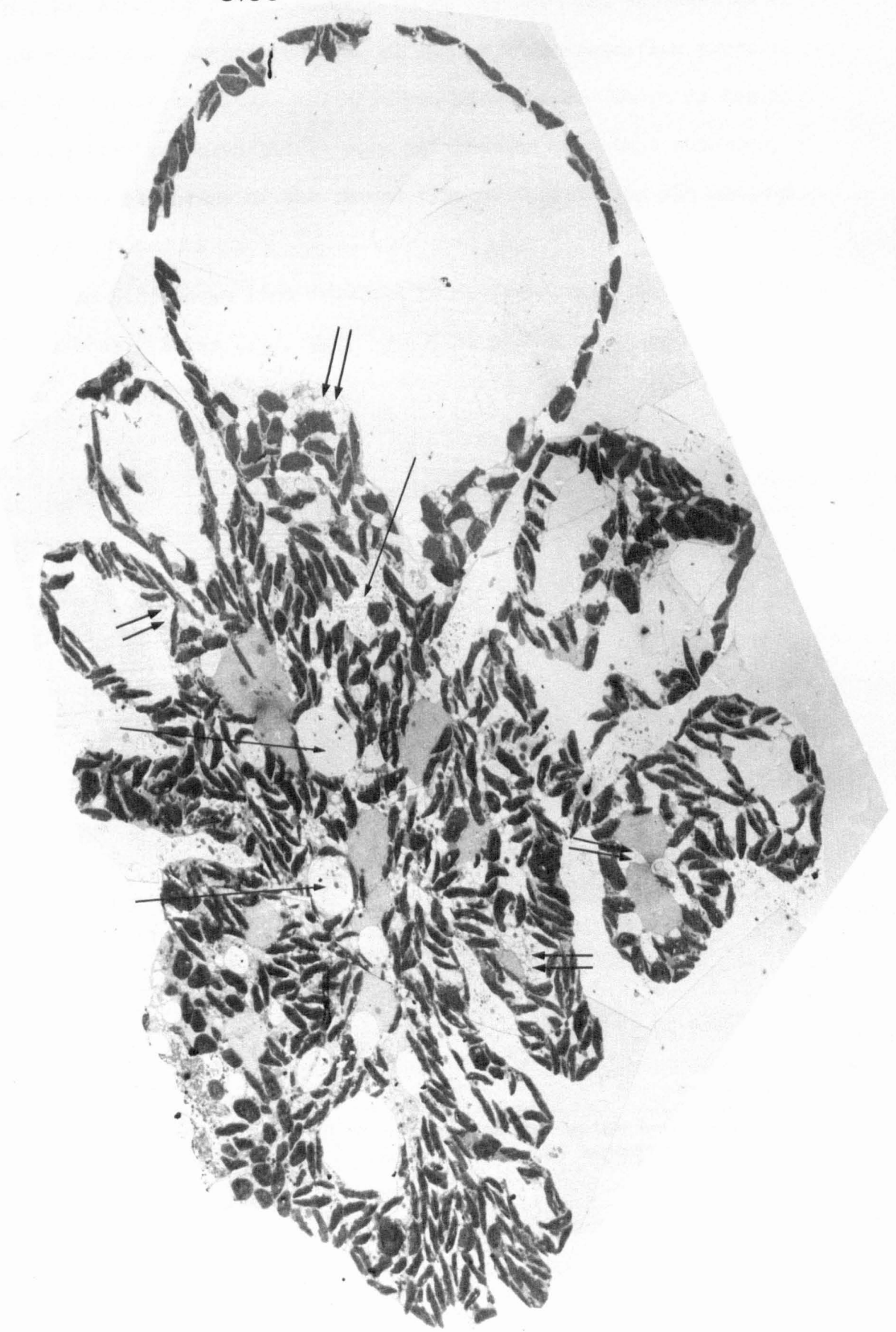


Line drawings taken from serial sections through a tobacco mesophyll spontaneous fusion body. x 400, section spacing approx. 20 μ m.

Figure 3.85 An electron micrograph(montage)of a section through a tobacco mesophyll spontaneous fusion body.The section corresponds to section No.3 in Figure 3.83. The highly cytoplasmic mass is dissected by deep clefts which contain cytoplasmic fragments. Vacuoles, probably transversely cut clefts, are arrowed. Regions of vesiculation, adjacent to vacuoles are indicated by double arrows. Details of regions of the fusion body are shown in Figures 3.86 to 3.89.

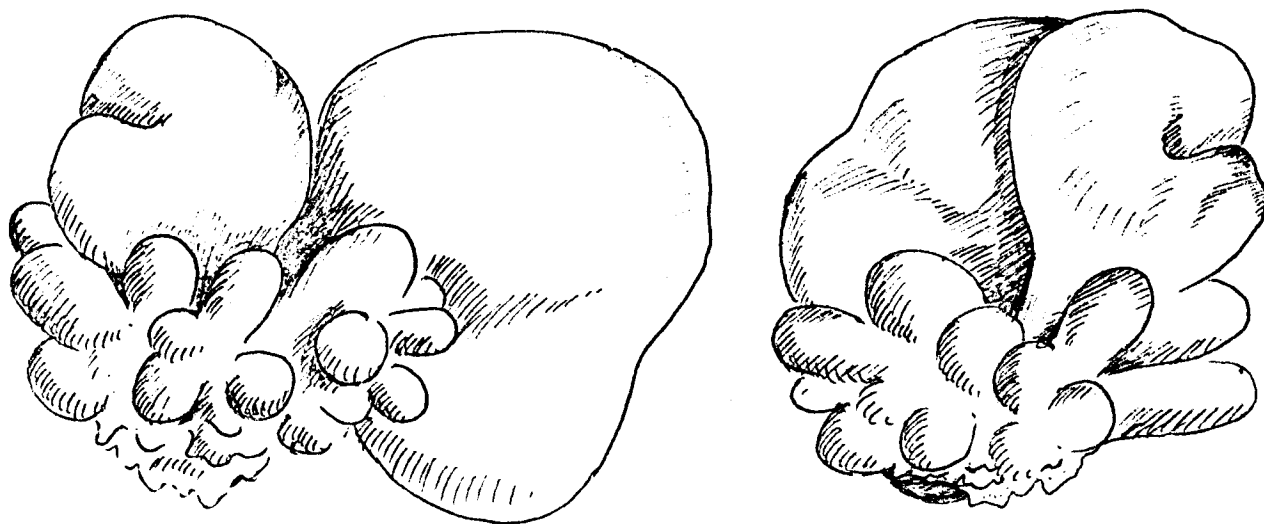
x 1,500

3.85



during the rounding off process, a study of fixed and embedded material was carried out. Serial sections of fusion body containing preparations were observed in the light and electron microscope. Those of the first type described revealed little more information when thus observed, however, the structure of the second type of fusion body was greatly clarified.

Figure 3.83 shows line drawings taken from serial sections through a fusion body. Below (Fig. 384) are perspective drawings reconstructing the fusion body from the sections.



(x 450)

Figure 3.84 Reconstructions of a spontaneous fusion body undergoing rounding off. Views along the two major axes are shown.

A thin section, corresponding to section number 3 in Figure 3.83, was examined in the electron microscope. Figure 3.85, a montage of four

electron micrographs covering the section, demonstrates the polarity of the fusion body, although the section does not pass centrally through the largest vacuole.

The highly cytoplasmic end of the body is dissected by deep clefts containing membrane bound cytoplasmic fragments and occasionally mitochondria. (Fig. 3.86). Irregularly shaped and rounded vesicles (Fig. 3.85 arrows), also containing the cytoplasmic fragments, probably represent transversely cut clefts. The outer surface of the fusion body is marked by very little particulate material.

There is evidence of considerable but localised damage on the periphery of the fusion body, corresponding perhaps to the arrowed region of Figure 3.82b. Figure 3.87 shows this at a higher magnification. A clear limiting membrane is lacking, the outer surface being marked by undulations and dense material. "Bubbles" of membrane lie some distance outside the body. Bundles of fibrous material are evident (arrows) on and near the outer surface. A number of chloroplasts lying just inside the body are diffuse and lack an outer membrane but a dense irregular thick line marks their outermost surface. It is noted that in this stage the grana and stroma lamellae of the chloroplasts can be seen very clearly. This would appear to be the limit of the cytoplasmic damage, thus demonstrating a rather effective sealing mechanism.

The tonoplast region of the large vacuole shows apparent activity where it adjoins the cytoplasmic mass. (Fig. 3.88). Many small vesicles lie between the large organelles and the vacuole. Such regions of vesiculation mark several of the smaller vacuoles in the body. (Fig. 3.85, double arrows). The mass of cytoplasm contains some dispersed small vesicles but no large vacuoles.

Ultrastructural features of tobacco
mesophyll spontaneous fusion bodies

Figure 3.86 The contents of one of the clefts which dissect the cytoplasmic mass. Membrane bound fragments of cytoplasm and a mitochondrion (m) can be seen.

x 35,000

Figure 3.87 Part of the damaged region of the fusion body, (bottom left in Fig. 3.85). Degenerating chloroplasts (c) are limited on the outer side by a dense irregular line of amorphous material. Bundles of fibres (arrowed and illustrated below) can be seen outside this line. 'Bubbles' of membrane lie outside the fusion body.

x.30,000

Figure 3.88 A region of vesiculation similar to that adjoining the large vacuole in Figure 3.85.

x 15,000

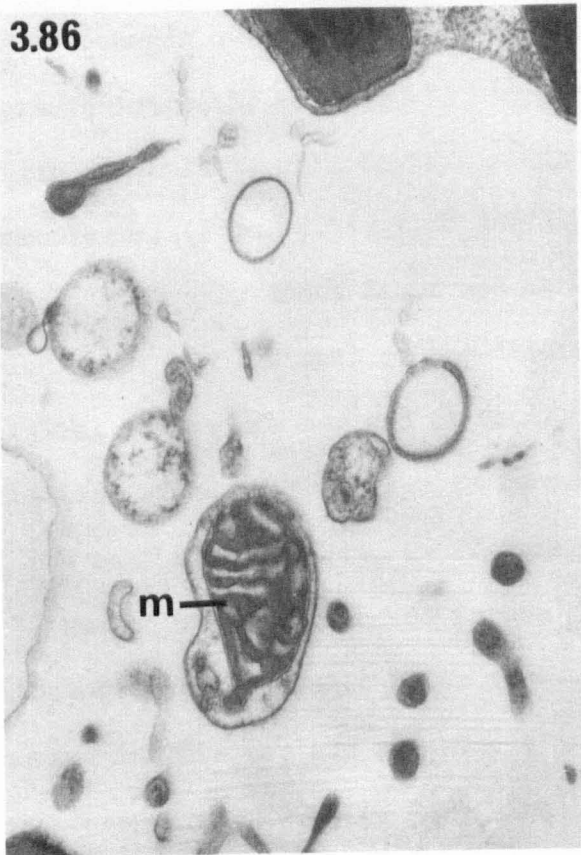
Figure 3.89 Four nuclei located near the centre of the cytoplasmic mass.

x 6,000

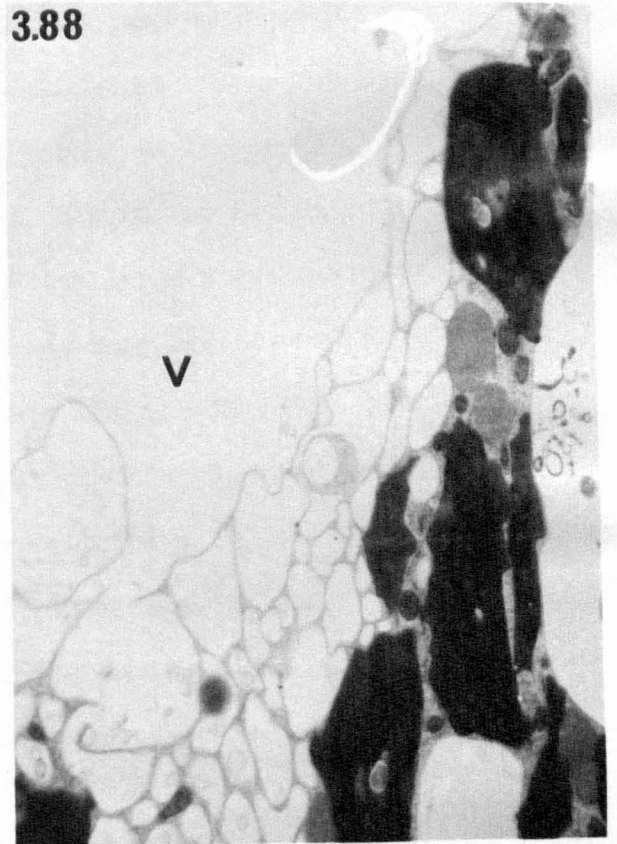


Detail from Fig. 3.87
(f=fibres)

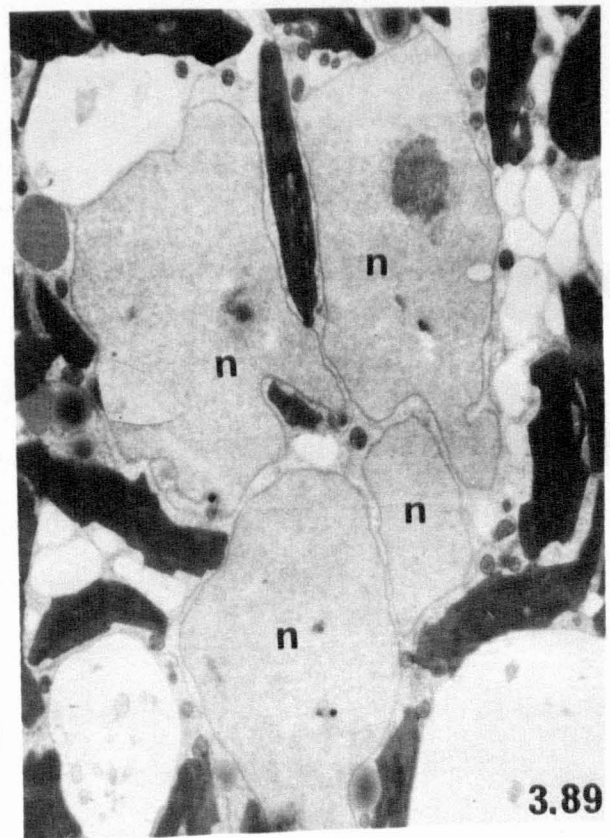
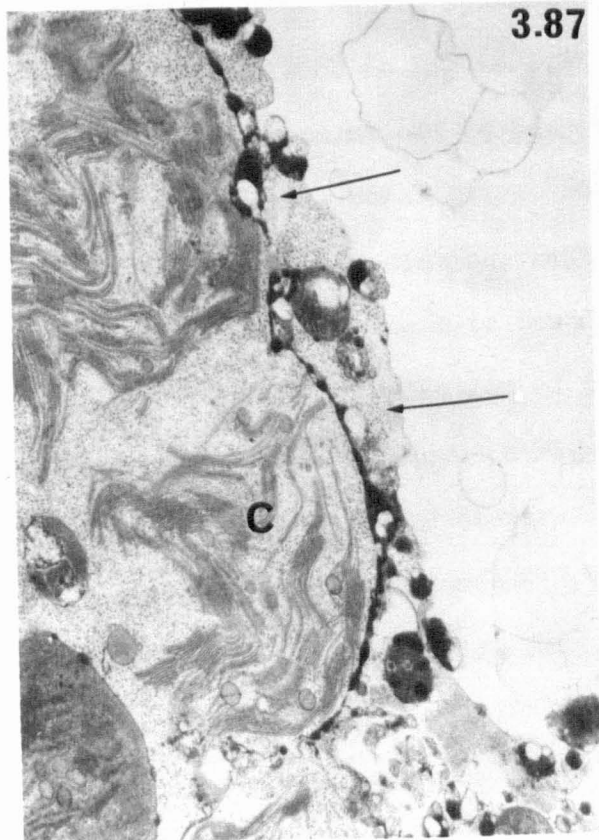
3.86



3.88



3.87



3.89

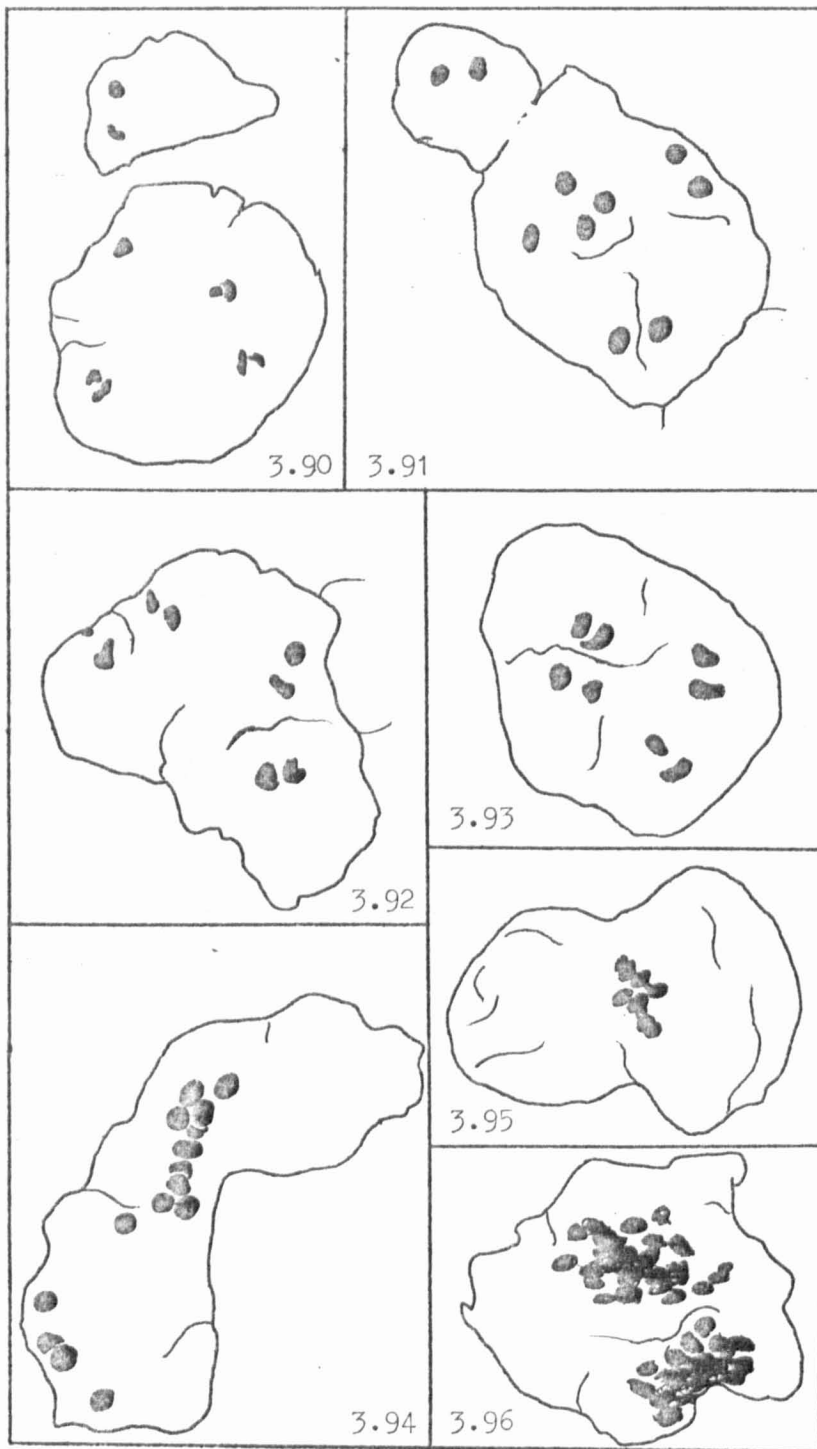
There is a more or less even distribution of chloroplasts and mitochondria in the cytoplasm, the latter however becoming characteristically orientated. In the undigested tissue and in the single protoplast, chloroplasts generally tend to lie parallel to the plasmalemma, (Figs. 3.51, 3.68 and 3.76) but in the fusion body shown in Fig. 3.85 only those dispersed around the large vacuole are so orientated. In the rest of the fusion body they lie several deep parallel to one another and at an angle to the plasmalemma. The direction of their long axes is generally pointing towards the centre of the mass of cytoplasm, as if some centripetal flow were taking place.

Nuclei are interspersed in the cytoplasm, some obviously associated with lobes of the fusion body. Many of the nuclei appear grouped, showing in this section six single nuclei, six associated in pairs and one group of four. (Fig. 3.89 also).

In terms of the entire spontaneous fusion body, the interpretation of a thin section such as Figure 3.85 is rather limited. In contrast, Feulgen stained preparations do demonstrate the nuclear positions in whole fusion bodies. Small fusion bodies with 6 - 8 nuclei show a tendency to nuclear association, particularly pairing (Figs. 3.90 - 3.93). Large fusion bodies are seen to round off in two ways as described. This is reflected in the distribution of nuclei. Figure 3.94 corresponds to the type of fusion body shown in Figure 3.80. Some nuclei are concentrated centrally whilst others are dispersed. The second type of fusion body with clearly defined vacuolar and cytoplasmic regions has a tight concentration of nuclei, (Fig. 3.95) or where a very large number are involved, more than one concentration. (Fig. 3.96).

The technique does not show the large fusion bodies to advantage, it being difficult to visualise all of the nuclei in one plane without

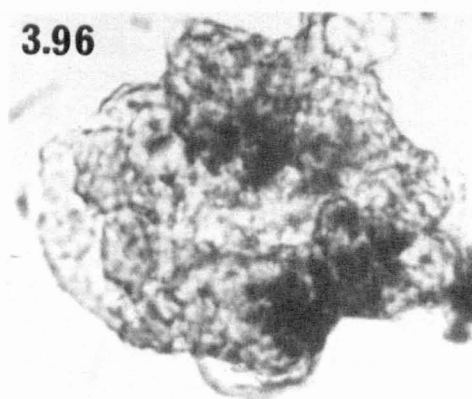
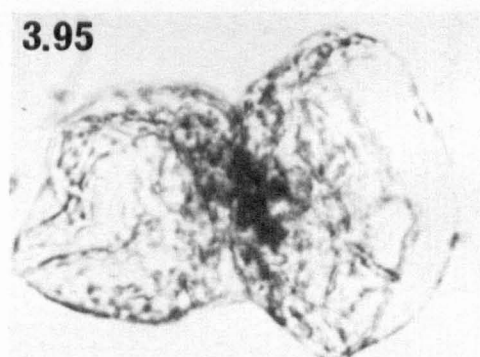
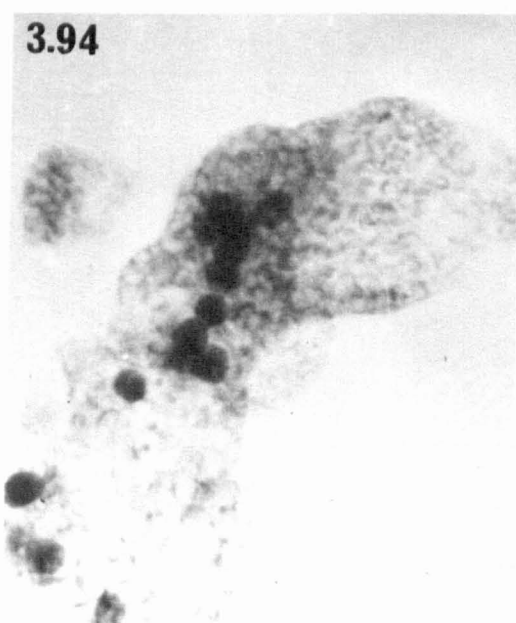
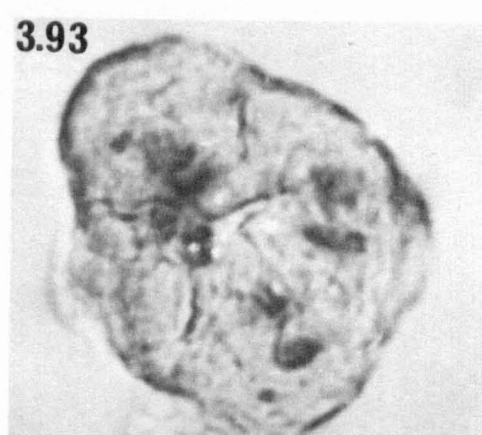
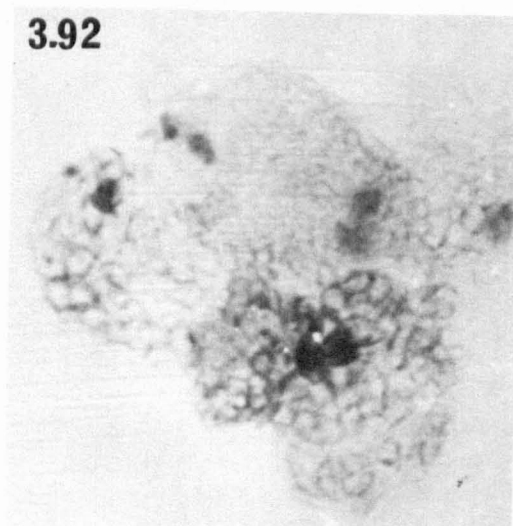
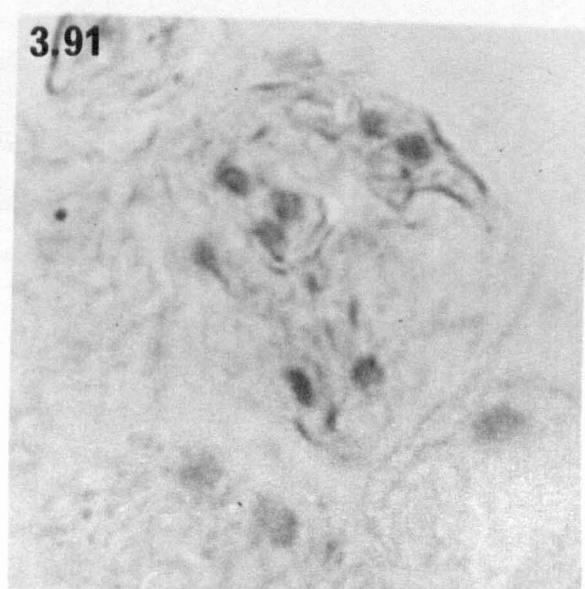
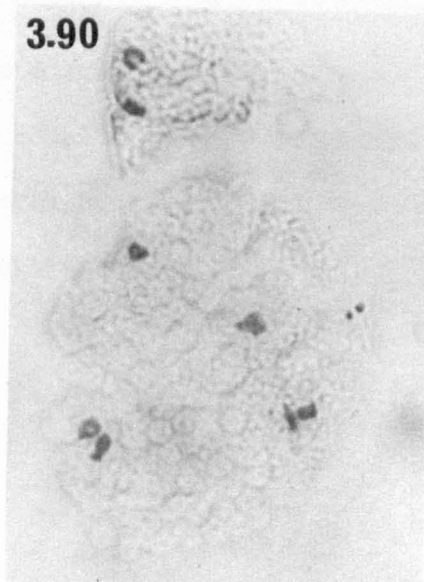
The distribution of nuclei in tobacco mesophyll
spontaneous fusion bodies



Figure

Magnification

3.90	x 550
3.91	x 1,000
3.92	x 850
3.93	x 1,000
3.94	x 1,200
3.95	x 800
3.96	x 800



greatly disrupting the structure of the bodies by squashing them.

A detailed study of nuclear associations and other fine structural considerations is included in aspects of fusion body culture. (Section 3.4).

Summary

(The tobacco leaf system)

The structure of the tobacco leaf and the stages of enzyme digestion of the leaf tissues are described, drawing on light and electron microscopical evidence. A relationship between the structure of the fusion bodies released and the original tissue structure is suggestive of an involvement of plasmodesmata in the spontaneous fusion process. Electron microscopic observations of stages in fusion body formation support this.

It is observed that a considerable amount of vesiculation of the protoplast cytoplasm occurs during enzyme treatment and the accompanying plasmolysis. This appears to result from an invagination of the plasmalemma and a pinching off of plasmalemmar vesicles. The process facilitates the uptake of extracytoplasmic material.

Changes occurring in spontaneous fusion bodies following isolation are described. The structure of a partially rounded-off spontaneous fusion body is investigated using light microscopic serial sectioning and electron microscopy. Processes of nuclear aggregation and the reorganization of cytoplasmic organelles and vacuoles are described.

3.4 THE CULTURE OF TOBACCO MESOPHYLL PROTOPLASTS AND SPONTANEOUS FUSION BODIES.

3.4.1 Solid Culture.

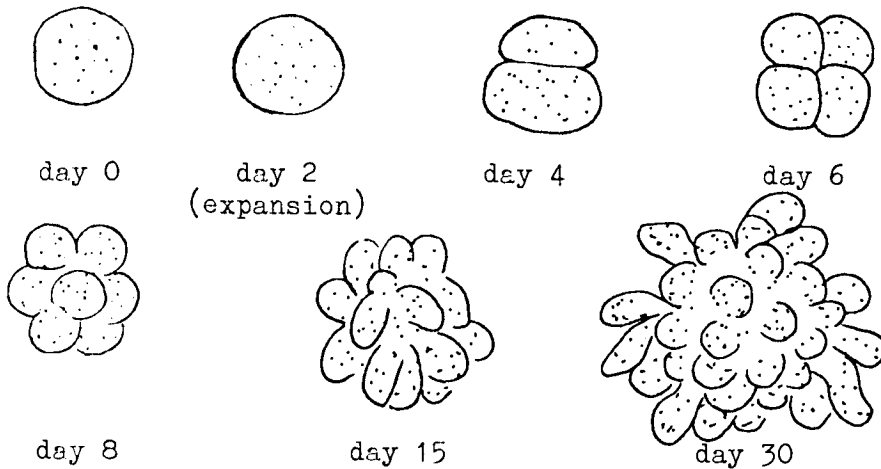
Cultures of uninucleate protoplast preparations in both White's and TMS medium, gave a fairly consistent pattern of wall regeneration and division. (Figs. 3.97 - 3.101). The micrographs cover a period of 1 to 30 days in culture, from an original density of 1×10^5 protoplasts per ml. In contrast spontaneous fusion bodies form a far from uniform population and therefore individual bodies were followed in culture. (These were located by marking the culture dish.)

Figures 3.102 to 3.105 show changes occurring in 4 fusion bodies over the first 6 days in culture. The cultures are in TMS medium at an original culture density of 0.5×10^5 protoplasts per ml. Individual details of isolation and culture are given in the legends. A slight expansion, rounding in outline and redistribution of organelles were generally observed. Figures 3.104 a to c, show a contrasting pattern but this body is considered anomalous. Generally the green pigmentation of the fusion bodies was replaced by a yellow brown colouration, evident also in the vacuoles and culture medium.

An apparent clearing of organelles from crescent shaped peripheral regions was observed (Figs. 3.103c and 3.105b). (extreme examples are shown in Figures 3.106 and 3.107, the former structure being rather dissociated from the main body and possibly subdivided. A vacuolated outpushing from the fusion body in Figure 3.102b is seen to flatten against the outer surface. (Fig. 3.102c). It appears to subdivide and become darker and granulated resembling the main body. (Fig. 3.102d) Calcofluor tests of such fusion bodies showed a general fluorescence over the surface with no localised areas of increased fluorescence.

The culture of tobacco mesophyll protoplasts

Figure 3.97. Typical stages in the regeneration of
a colony from a single tobacco mesophyll
protoplast. (Solid medium). x c.200

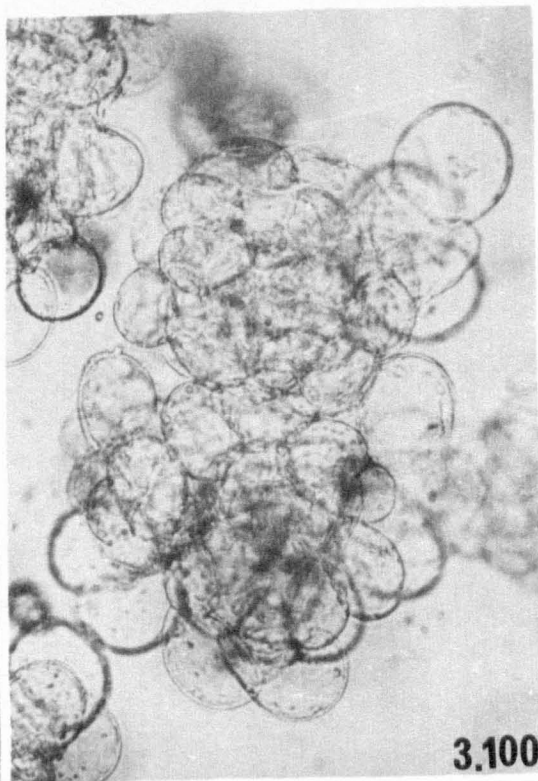


Figures 3.98 to 3.101. Light micrographs of some of the above stages.

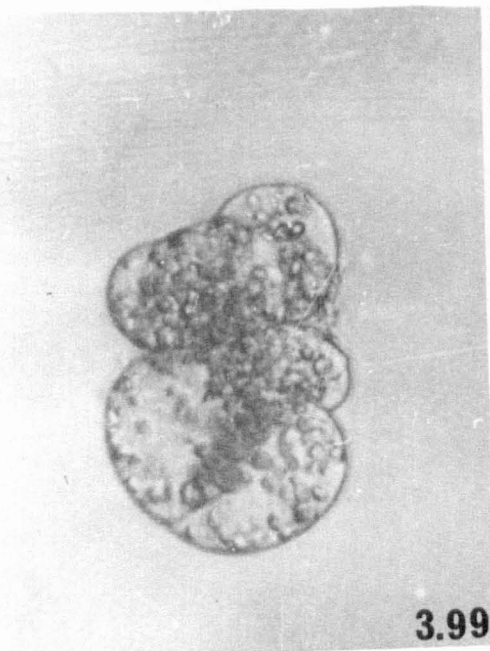
- | | | |
|--------------|---|-------|
| Figure 3.98 | Two four day dividing protoplasts. | x 350 |
| Figure 3.99 | An 8 day dividing protoplast. | x 350 |
| Figure 3.100 | Two colonies formed in a 28 day preparation. | x 300 |
| Figure 3.101 | A Calcofluor stained preparation viewed in UV light.
16 day preparation. | x 300 |



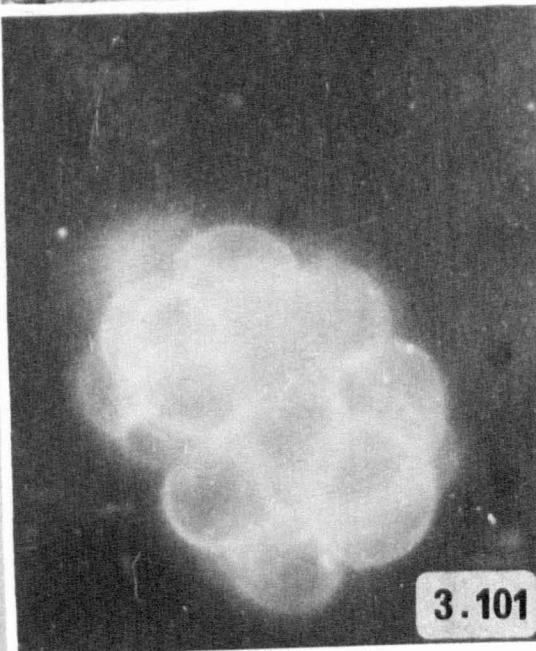
3.98



3.100



3.99



3.101

The culture of tobacco mesophyll spontaneous fusion bodies(solid cultur

(All fusion bodies isolated in an enzyme incubation medium containing 0.4% Macerozyme, 4% Meicelase and 13% sorbitol. All Figures x 125)

Figure 3.102 A fusion body showing extreme systrophy at day 0.

With time the organelles redistribute to cover the depleted vacuolar areas. An outpushing forms, which gradually merges with the rest of the fusion body.

a day 0

b day 2

c day 4

d day 6

Figure 3.103 The organelle redistribution occurs here also.

However, a clearing of organelles from a peripheral region is observed at 6 days.

a day 0

b day 2

c day 6

Figure 3.104 The fusion body appears to be degenerating.

a day 0

b day 4

c day 6

Figure 3.105 A fusion body similar to Figure 3.102. Some slight plasmolysis is observed at 6 days.

a day 3

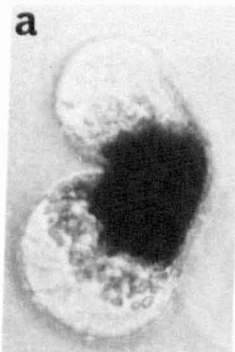
b day 6

Figures 3.106 and 3.107 Peripheral regions of the bodies are cleared of organelles and appear partitioned from the main body.

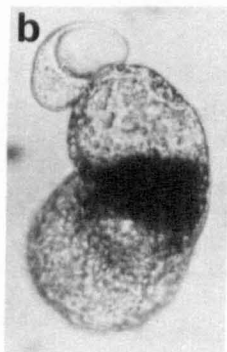
Figure 3.108 An irregularly shaped fusion body.No changes were observed over four days in culture.

3.102

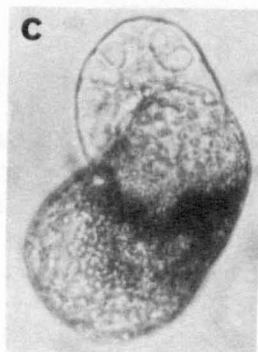
a



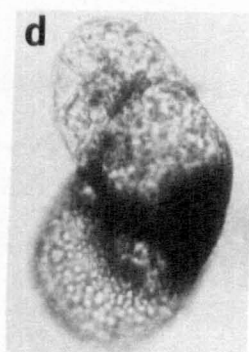
b



c

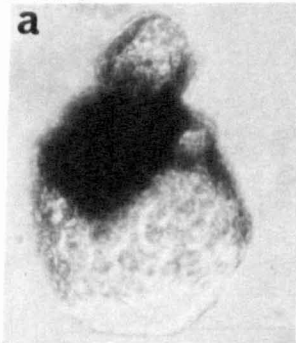


d

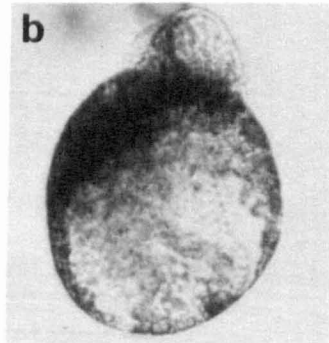


3.103

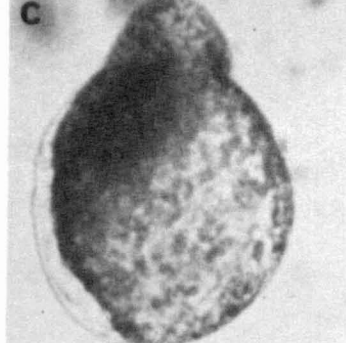
a



b

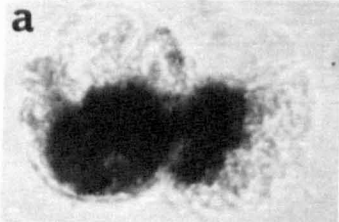


c

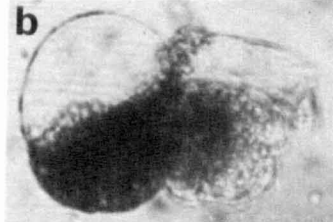


3.104

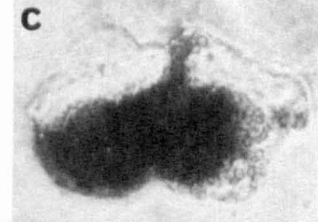
a



b

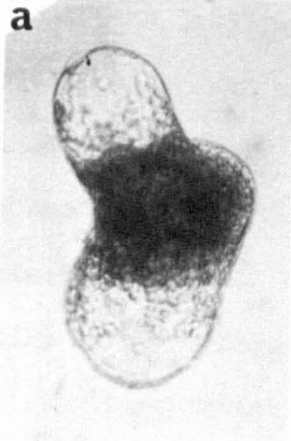


c

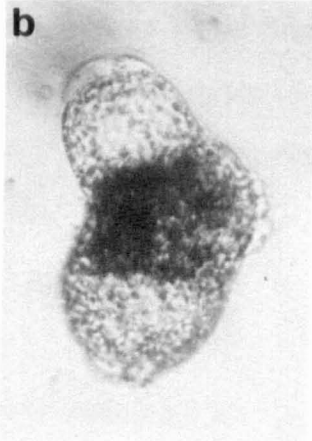


3.105

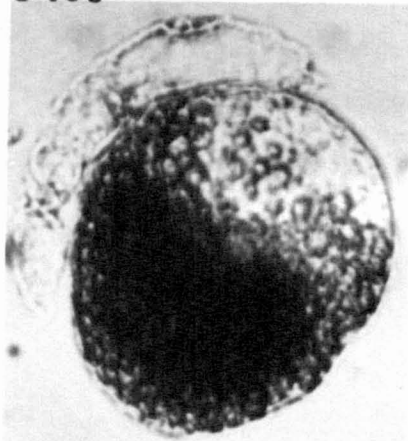
a



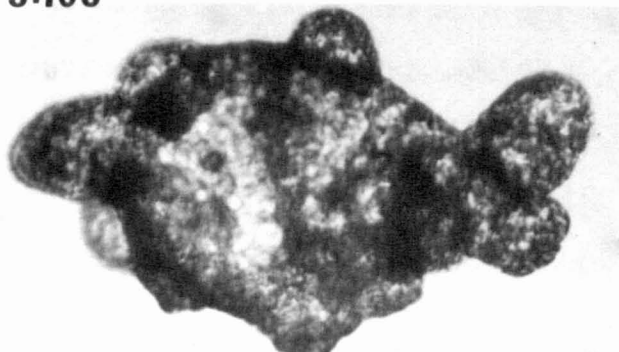
b



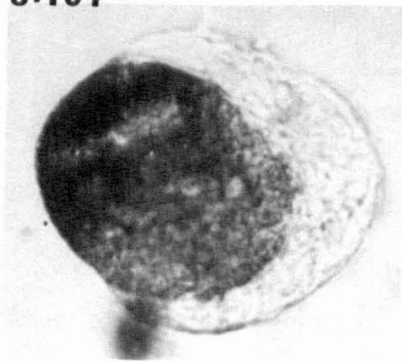
3.106



3.108



3.107



Irregularly shaped, larger fusion bodies (Fig. 3.108) changed little over the period in culture and none of the fusion bodies showed any further development or signs of division.

Some electron microscopy was carried out on solid cultures, but due to poor fixation and the low concentration of material, this was discontinued.

3.4.2 Liquid culture: light microscopic observations.

All liquid cultures were carried out in TMS medium containing between 10% and 16% w/v sorbitol and at a culture density of 1×10^5 for control, uninucleate preparations and 0.5×10^5 for spontaneous fusion body containing preparations. Small samples were removed aseptically at intervals and observed. The descriptions are taken from several cultures, details being given in the legends.

Rounding off took up to 2 days, being more rapid in low osmotica. (Figs. 3.109 to 3.111). At 4 days some fusion bodies became somewhat elongate in shape and a bubbling of cytoplasm and vacuole was commonly observed. (Figs. 3.112 to 3.114). Uninucleate protoplasts expanded a little but remained spherical. These entered their first division at approximately 7 days. Wall regeneration as revealed by Calcofluor occurred generally at 6 - 8 days.

Little further activity occurred in the fusion bodies between 4 and 7 days, after which time, three main patterns of behaviour were observed, one a continuation of the bubbling effect noted above. Clusters or chains (Fig. 3.115) of bubbles formed at one end of the fusion body. Some lacked any dense cytoplasmic content, (Figs. 3.116 and 3.117, arrows), but others, particularly in cultures with a low osmoticum level, were incompletely separated from the main body and clearly contained organelles. (Figs. 3.118

The culture of tobacco mesophyll spontaneous fusion bodies
liquid culture

(Fusion bodies isolated as described on preceding legend.

all figures x 200)

Figure 3.109 Day 0 fusion bodies in 16% sorbitol.

Figure 3.110 Day 2 fusion bodies in 16% sorbitol. They have rounded off but are still systrophied.

Figure 3.111 Day 2 fusion bodies in 10% sorbitol. There is no sign of systrophy.

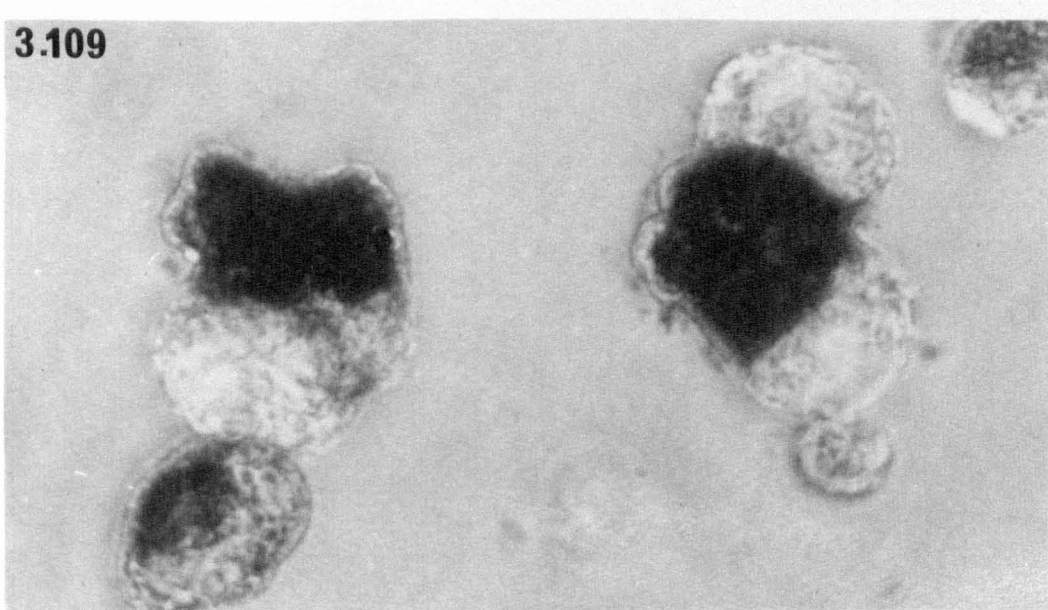
Figure 3.112 Day 3 fusion body with small protrusions. (in 16% sorbitol)

Figure 3.113 Day 4 fusion body in 13% sorbitol. The body is elongate and shows a vacuolar outpushing.

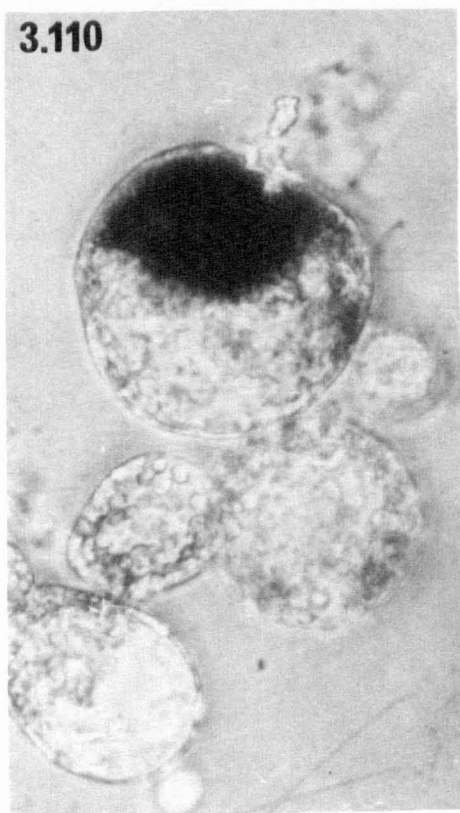
Figure 3.114 As above; with a more cytoplasmic outpushing.

(Phase contrast microscopy)

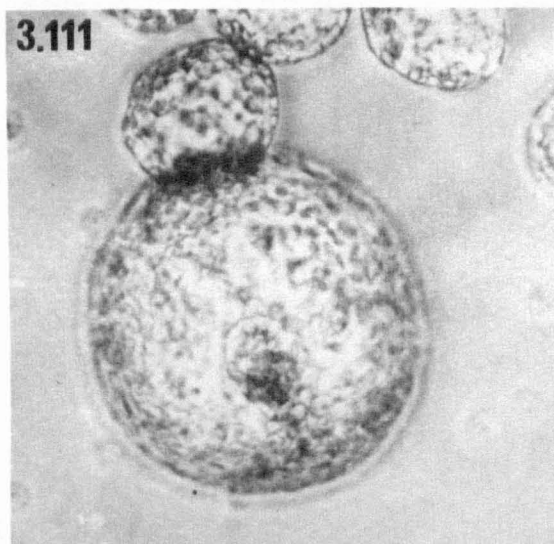
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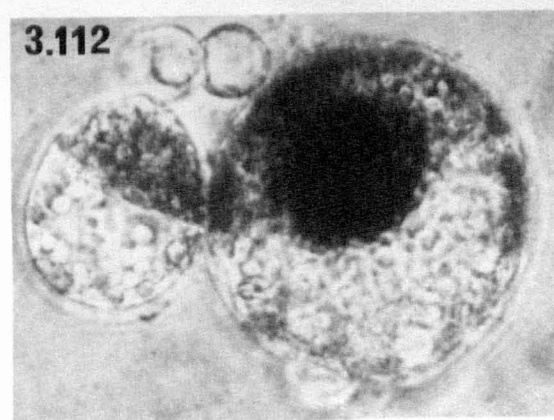
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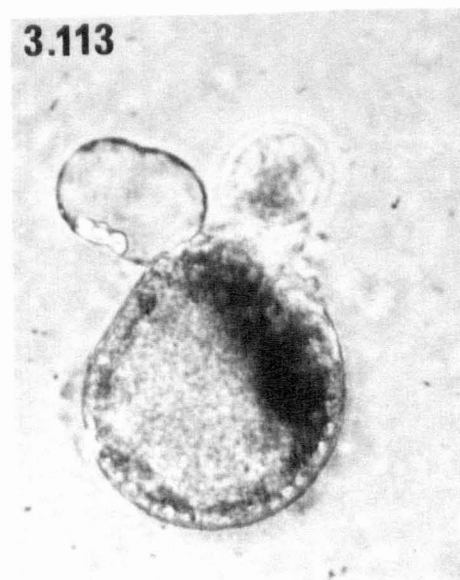
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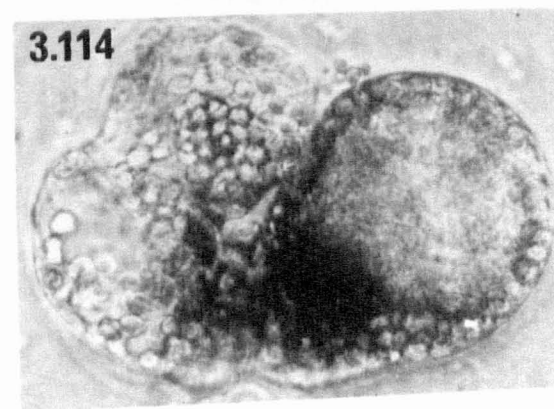
3.112



3.113



3.114



The culture of tobacco mesophyll spontaneous fusion bodies.

liquid culture

(Material isolated as already described. Figures following descriptions (bracketed) refer to number of days in culture and osmoticum levels.)

Figure 3.115 A group of spontaneous fusion bodies which have produced numerous protrusions, giving the impression of a colony.

(9,13%) phase x 150

Figure 3.116 A fusion body which has formed a mass of protrusions, some apparently free of organelles, others cytoplasmic.

(former type arrowed.) (11,10%) x 200

Figure 3.117 Two fusion bodies with organelle free protrusions.

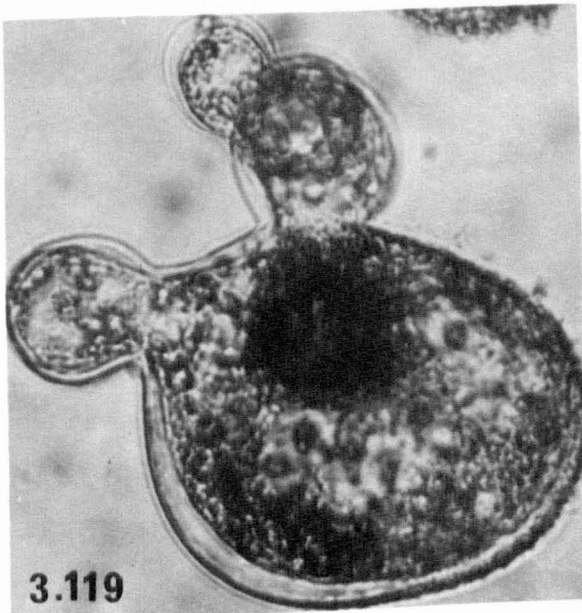
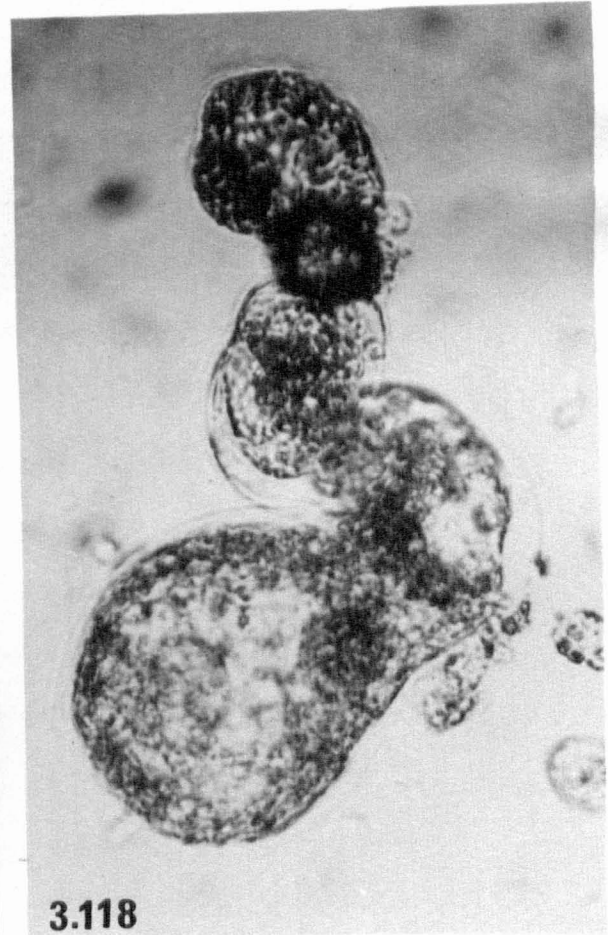
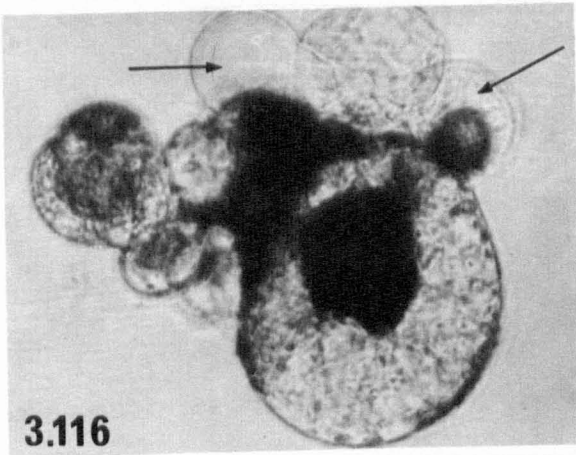
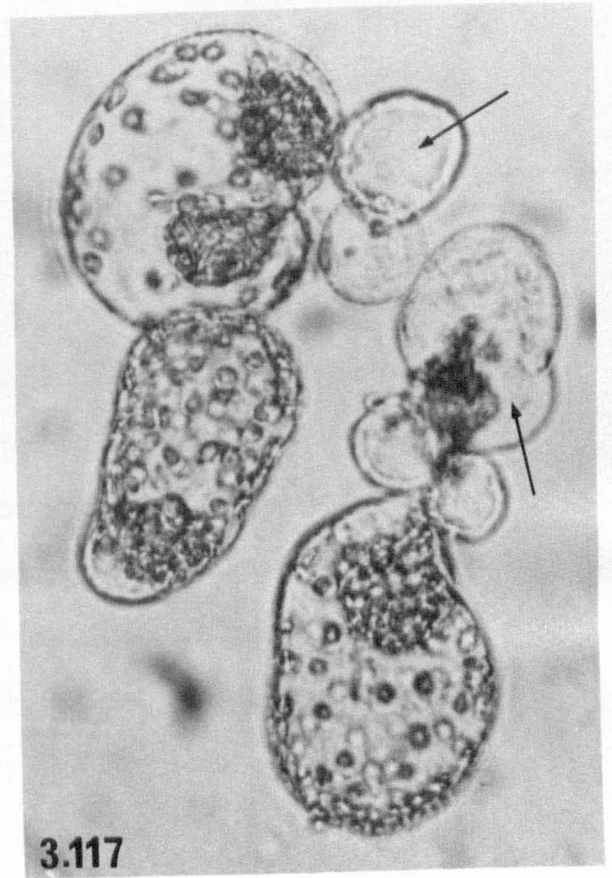
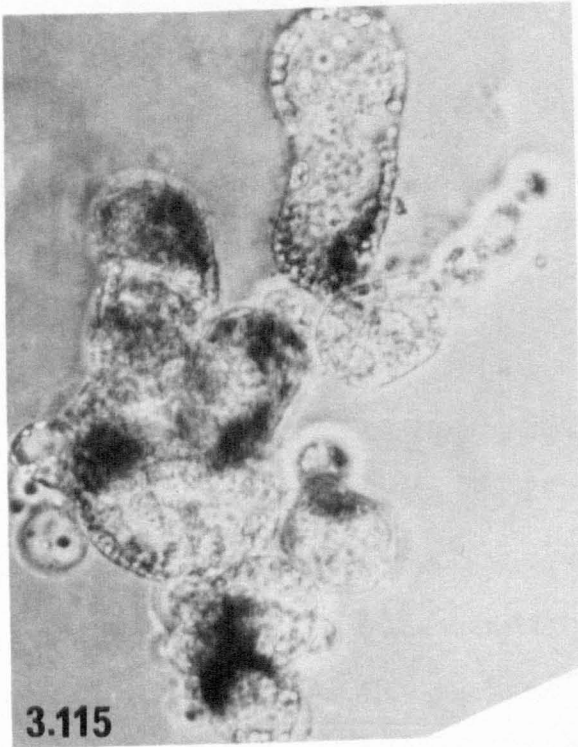
(arrowed). (13,10%) x 200

Figure 3.118 A chain of organelle containing protrusions forming from a fusion body.

(9,13%) x 200

Figure 3.119 Broad necked protrusions, containing organelles, extend from the somewhat plasmolysed fusion body.

(21,13%) x 225



and 3.119). This activity, plus the tendency of uninucleates to cluster and apparently adhere to fusion bodies, gave an appearance of early colony formation without any actual division occurring.

A division into two equal halves, resembling the normal pattern of division in uninucleates (Fig. 3.120 arrow) was occasionally observed. (Figs. 3.120 to 3.122). A marked aggregation of cytoplasm at the crosswall was apparent, particularly in older cultures which had assumed a brown colouration. In a high osmoticum the divisions were frequently incomplete, plasmolysis occurring. At a low osmoticum the division produced a dumbbell shaped structure. In Fig. 3.123 induced plasmolysis shows that here, division is again incomplete. Occasionally the division was oblique. (Fig. 3.124). Some fusion bodies both divided and showed the bubbling effect at one end. (Fig. 3.125).

A rather more commonly observed type of division was an asymmetrical partitioning of one part of the fusion body, frequently in the highly cytoplasmic region. (Figs. 3.126 and 3.127). A second, equal division was occasionally seen within this subdivision. (Figs. 3.128 and 3.129). The large vacuolate part of the body rarely showed any activity apart from localised plasmolysis. (Figs. 3.128 and 3.129 arrows). Other less regular divisions, tending to compartmentalise the fusion bodies were observed. (Figs. 3.130 to 3.132).

These changes occurred in 25 days of culture by which time many uninucleate protoplasts had undergone two to four divisions. The fusion body containing cultures declined from this time onwards, extreme darkening and plasmolysis occurring with an increase in the surface debris evident. Two cultures were Feulgen stained upon isolation, after 12/15 days and after 20/23 days in culture. Some 22% of multinucleates declined to approximately 9% and finally 3% over the culture period. (Details in

The culture of tobacco mesophyll spontaneous fusion bodies
liquid culture

(Material isolated as already described. Details of culture included as in previous figures.)

Figure 3.120 Division of a fusion body into two equal halves.(lower)

Above, a single protoplast is dividing similarly.

(arrowed) (11,13%)phase x 300

Figure 3.121 As Figure 3.120. (13,13%) x 350

Figure 3.122 As Figure 3.120. (23,16%) x 350

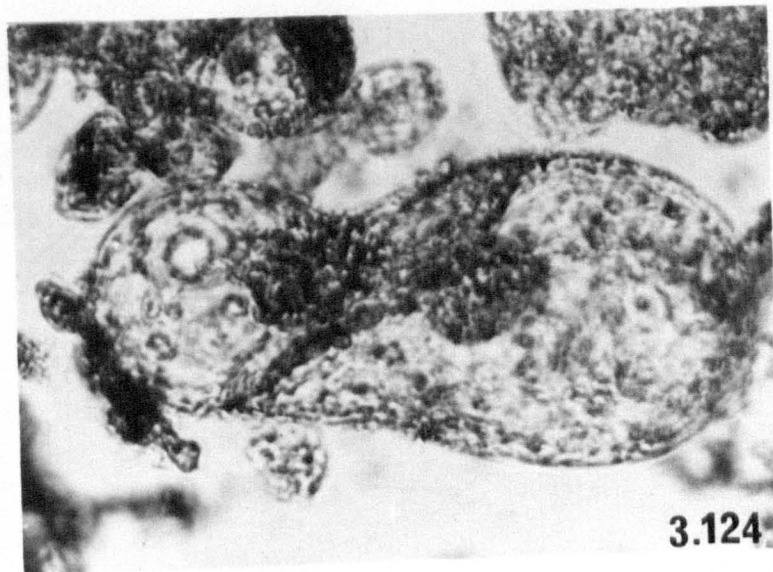
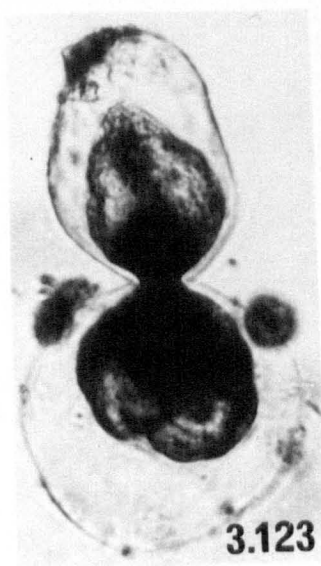
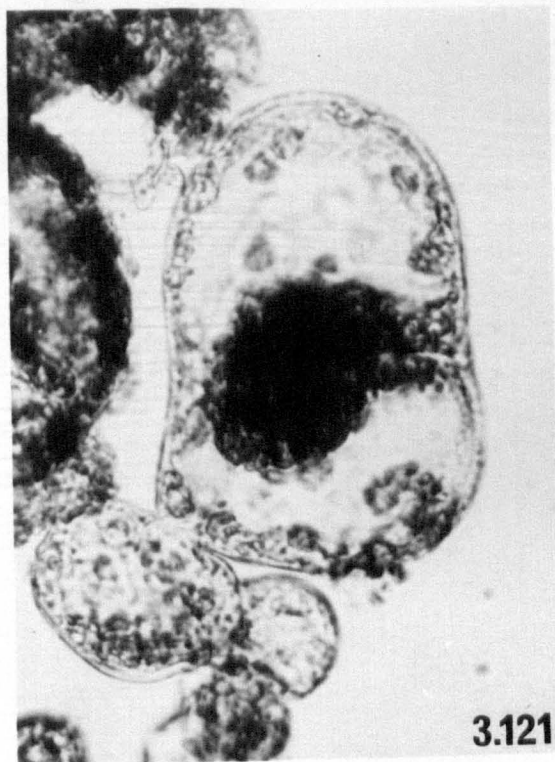
Figure 3.123 A fusion body which has begun to divide. The body was intentionally plasmolysed.

(9,10%) x 275

Figure 3.124 An oblique division. (9,13%) x 350

Figure 3.125 A dividing fusion body has a cluster of protrusions at one end. Some plasmolysis is occurring.

(22,13%) x 350



The culture of tobacco mesophyll spontaneous fusion bodies

liquid culture

(Material isolated as already described. Details of culture included
as in previous figures.)

Figure 3.126 A partitioning of the cytoplasmic end of the fusion
body has occurred. The division wall is arrowed.

(13,13%) x 275

Figure 3.127 As Figure 3.126. (16,13%) x 250

Figures 3.129 and 3.129 Second, equal divisions have occurred,
following an initial division such as that illustrated
above. Some plasmolysis is evident. (arrows)

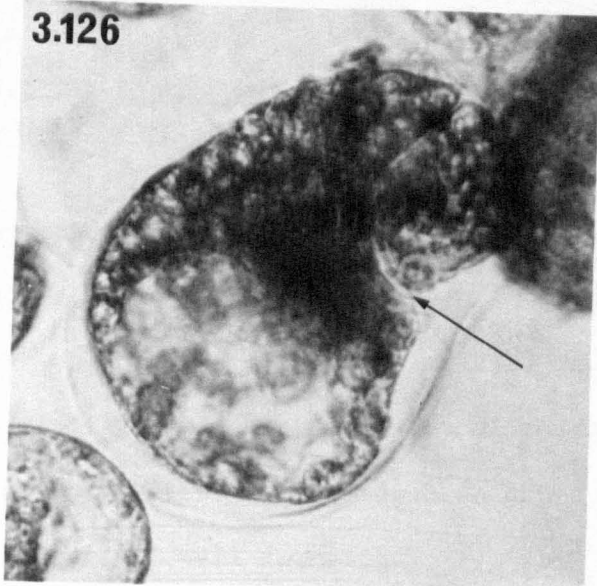
(20,13%) x 175

Figures 3.130 to 3.132 Irregular divisions in fusion bodies.

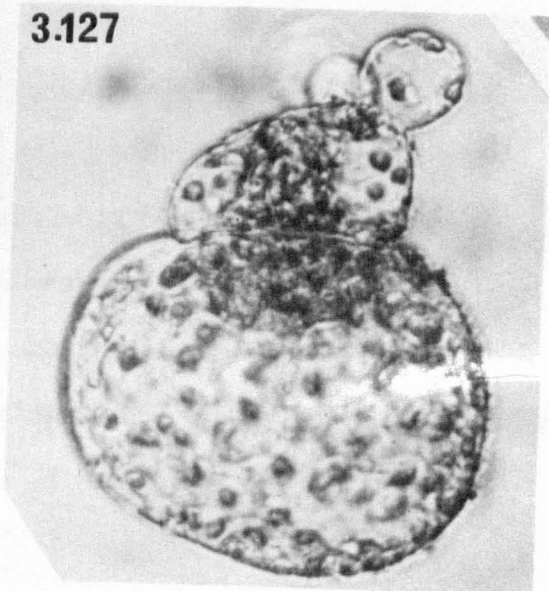
Figures 3.130 and 3.132 (13,13%) x 150

Figure 3.131 (22,13%) x 200

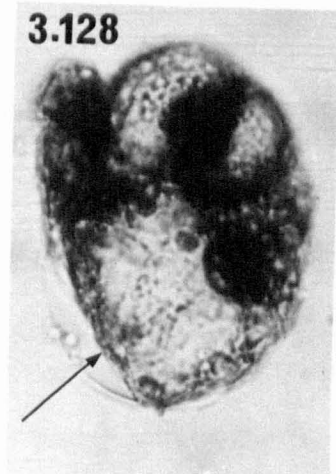
3.126



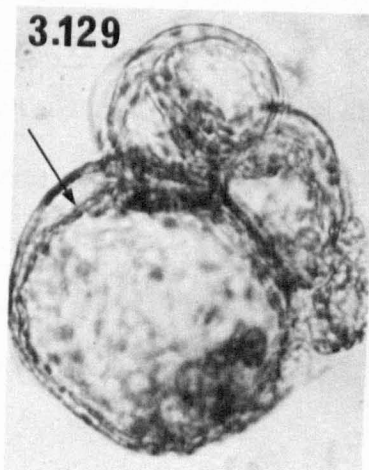
3.127



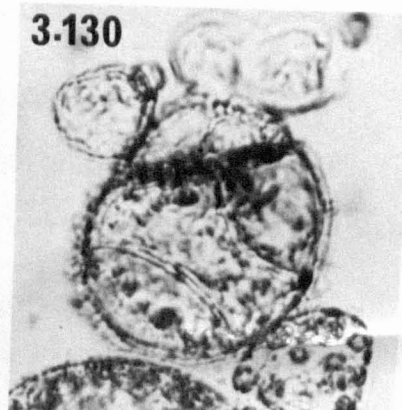
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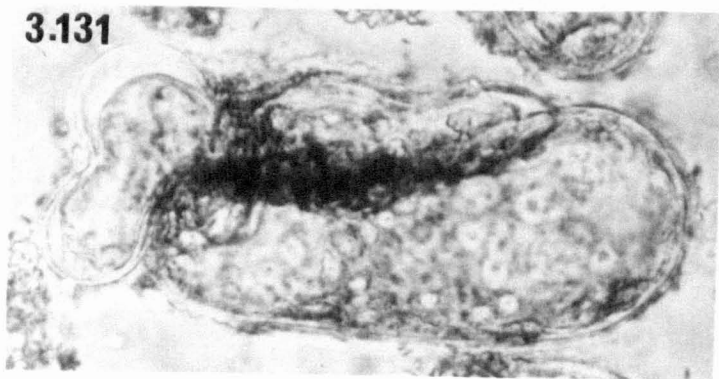
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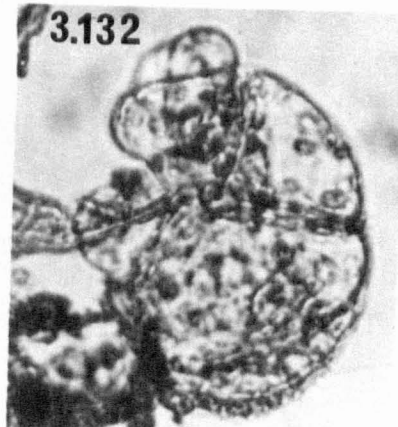
3.130



3.131



3.132



Section 3.5.4.

3.4.3 Liquid culture: electron microscopic observations.

Again samples were aseptically removed from control and spontaneous fusion body containing preparations. The observations cover a period from 1 to 14 days in culture after which period the latter cultures were heavily depleted and showing signs of senescence, not readily apparent upon light microscopic observation. Cytoplasmic changes are first described, then aspects of nuclear structure and behaviour and wall formation.

a. Chloroplasts

In the freshly isolated protoplast and fusion body, the chloroplasts are dense and irregularly shaped with straight sides broken by angular projections. (Fig. 3.133). The grana are centrally placed, being surrounded by fine, highly regular crystalline material. (Fig. 3.134). Little starch is present and very little peripheral reticulum can be seen. At 24 hours post isolation, uninucleate protoplast chloroplasts lose the angular outline and begin to accumulate starch. However, in the fusion bodies, although the latter is occurring the outline of the chloroplasts remains angular and there is a sudden increase in tubular, hexagonally packed peripheral reticulum. (Figs. 3.135 and 3.136a and b.)

At 4 days, the control preparations showed a great increase in starch and some decline in grana lamellae. (Fig. 3.137). In the fusion bodies the chloroplast population began to vary considerably, some storing a lot of starch and containing a peripheral reticulum of

The culture of spontaneous fusion bodies
Chloroplast structure

Figures 3.133 and 3.134 Chloroplasts in freshly isolated material.

They are irregular in outline, with crystalline material lying outside the grana, some forming angular projections. Little starch is present.

Figure 3.133 x 6,000

Figure 3.134 x 22,000

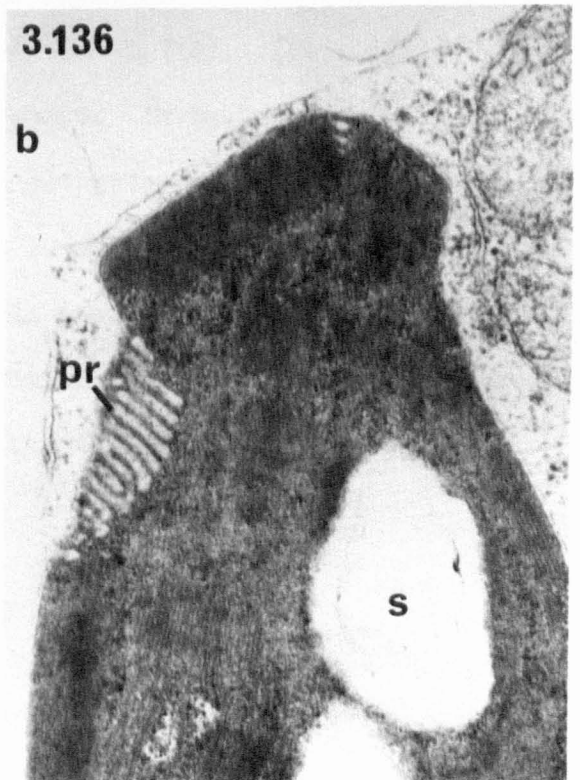
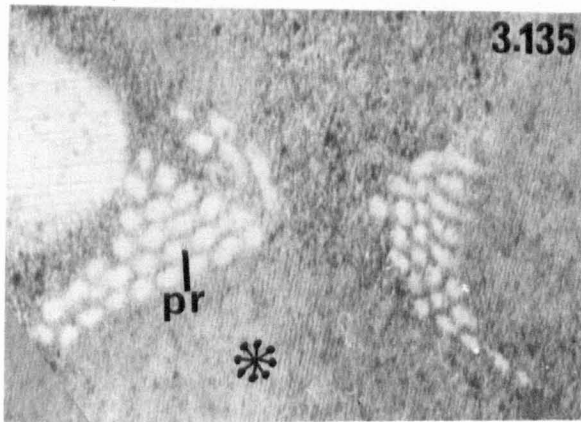
Figure 3.135 A region of a chloroplast in a spontaneous fusion body cultured for 1 day. The crystalline material is still evident (*) and there is a proliferation of hexagonally packed peripheral reticulum (pr). This is cut transversely in the section.

x 48,000

Figures 3.136 a and b As above. The peripheral reticulum is cut longitudinally, showing a tubular formation. Some starch is present.(s).

Figure 3.136a x 17,500

Figure 3.136b x 35,000



large vesicles. (Fig. 3.138). Others contained little or no starch and the peripheral reticulum was poorly developed if present. (Fig. 3.139 arrows).

Over the next 9 days, all preparations showed a differentiation of chloroplast structure. Generally the elongate shape became more rounded, accompanied in some chloroplasts by enormous starch accumulation, (Fig. 3.141) and a considerable reduction in both grana and stroma lamellae. (Figs. 3.140 and 3.141.) Some membrane figures in the cytoplasm suggested chloroplast breakdown, (e.g. Fig. 3.188 arrow) With time the chloroplasts became closely packed, generally remaining located in the nuclear region of the cytoplasm but occasionally bordering newly formed walls. (Fig. 3.179). The sizes of fusion bodies being very variable, no meaningful counts of chloroplast numbers could be made.

b. Mitochondria

Those in freshly isolated material showed a dark matrix common in plasmolyzed material. (Figs. 3.86 and 3.134), (Prat 1972). They are of uniform size and rounded in shape. In culture, the dense matrix is lost and they enlarge and elongate. An increase in numbers is noted with a tendency to localised accumulation of mitochondria near regions of new wall growth. (Fig. 3.187 and 3.188). In older cultures, asymmetrical division occasionally separated areas of cytoplasm rich in mitochondria from the rest of the cytoplasm. (Fig. 3.175).

c. Endoplasmic reticulum and ribosomes

Little change was observed in these organelles except for a location of e.r. and golgi like bodies near regenerating walls

The culture of spontaneous fusion bodies

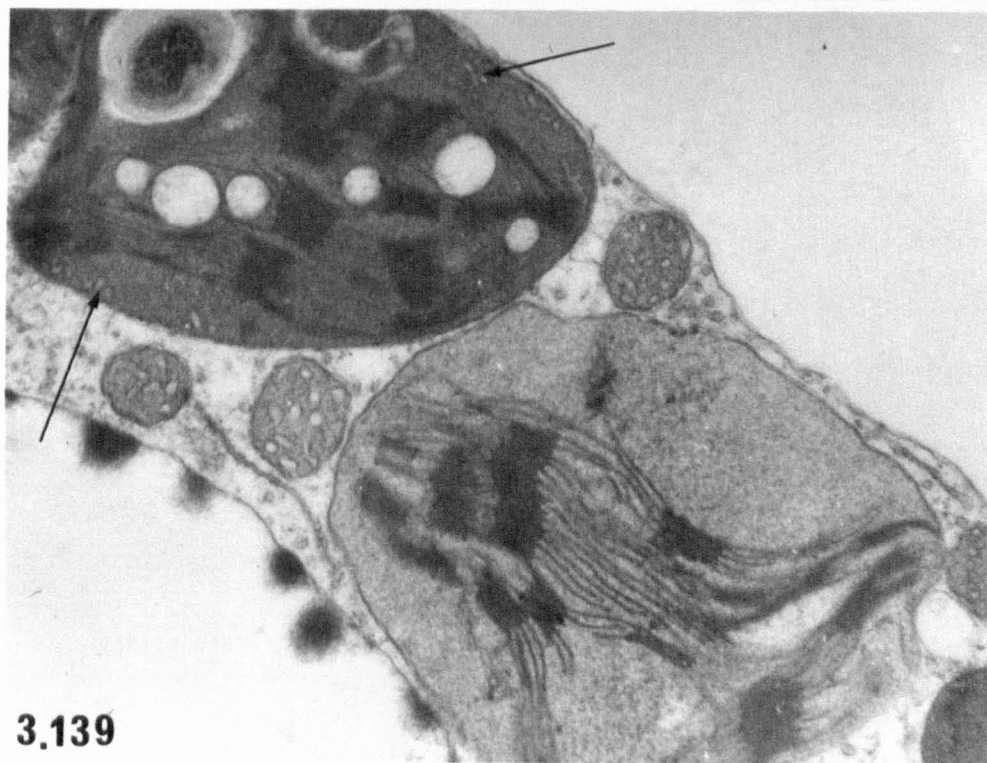
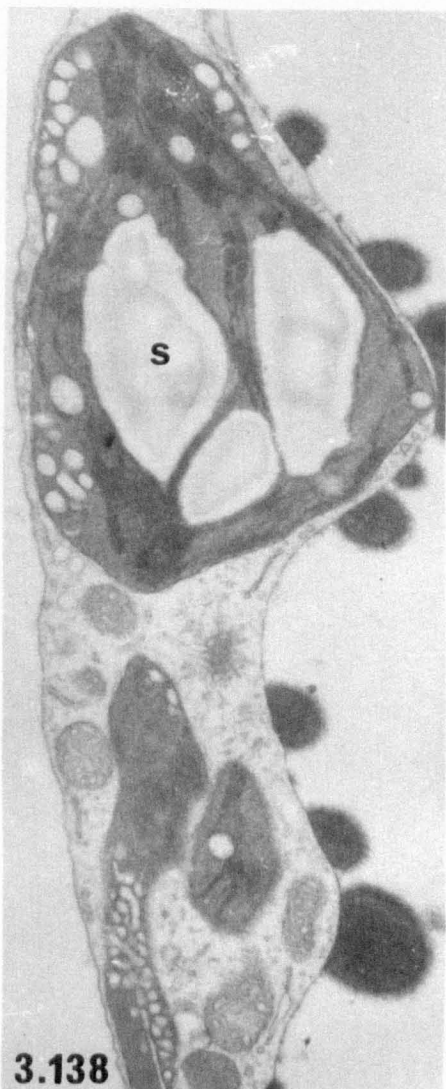
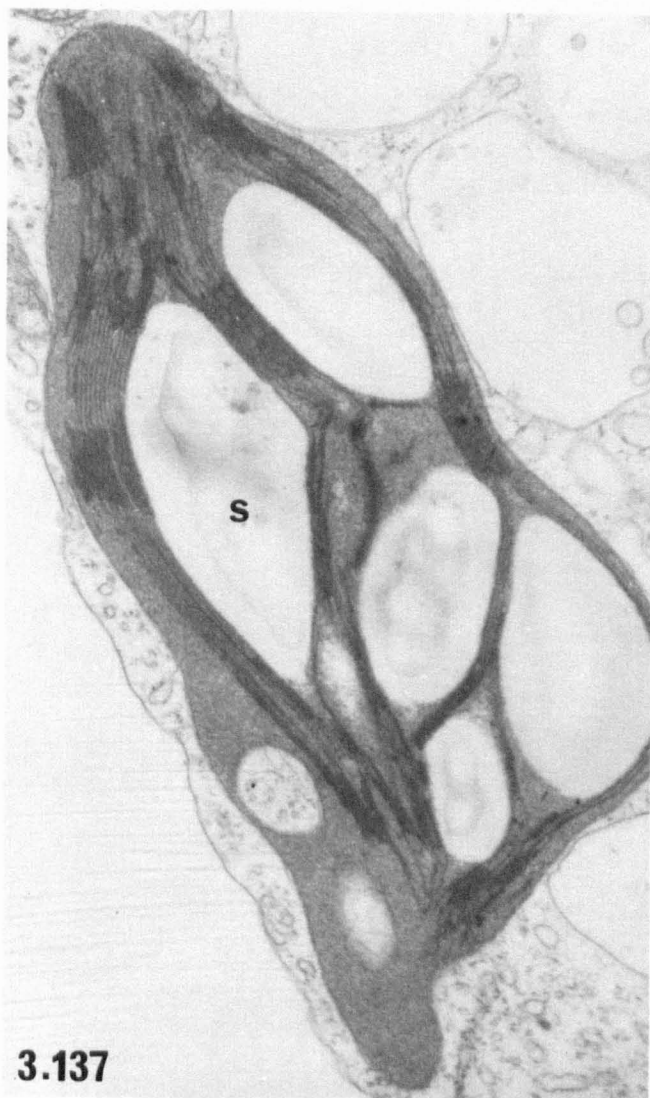
chloroplast structure

Figure 3.137 A chloroplast in a uninucleate protoplast cultured for four days. Much starch(s) has accumulated, separating and displacing the somewhat reduced grana. x 30,000

Figures 3.138 and 3.139 Chloroplasts in spontaneous fusion bodies after four days in culture . The hexagonally packed peripheral reticulum is lost. Some chloroplasts have a large vesiculate peripheral reticulum (Figure 3.138) and others very little at all(Figure 3.139 arrows.) Varying amounts of starch are stored. The grana and stroma lamellae show signs of reduction.

Figure 3.138 x 20,000

Figure 3.139 x 25,000

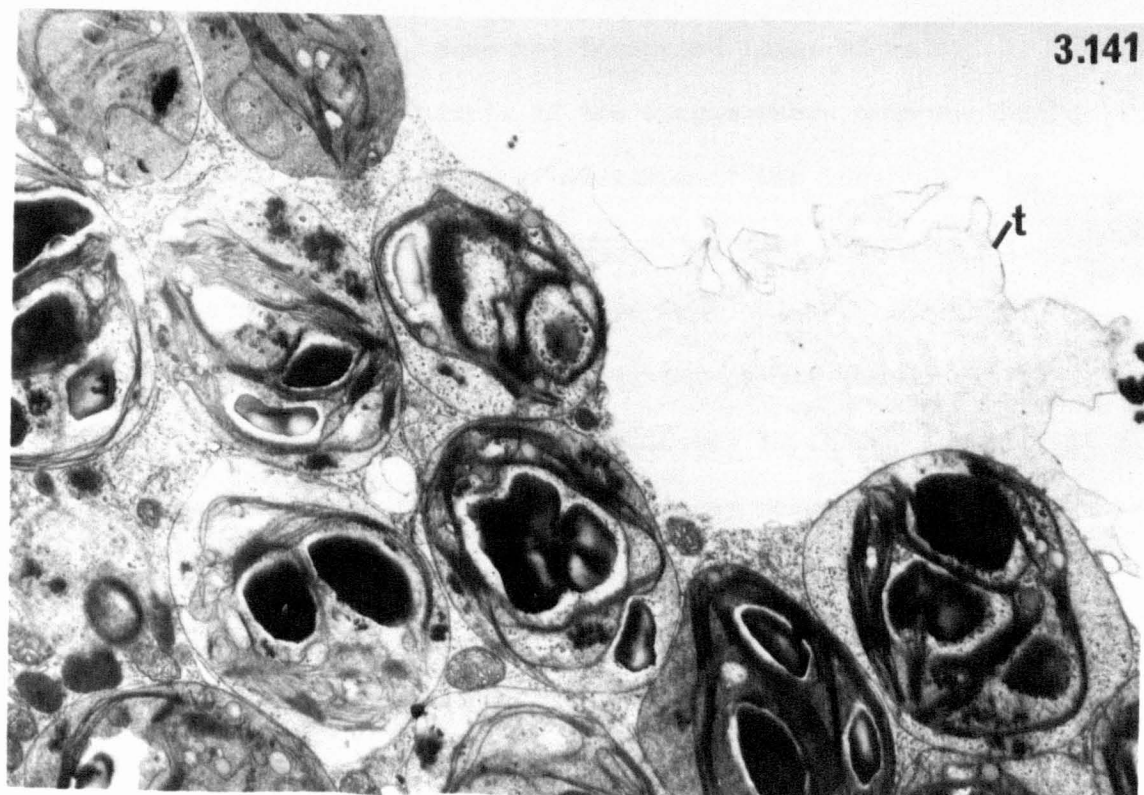
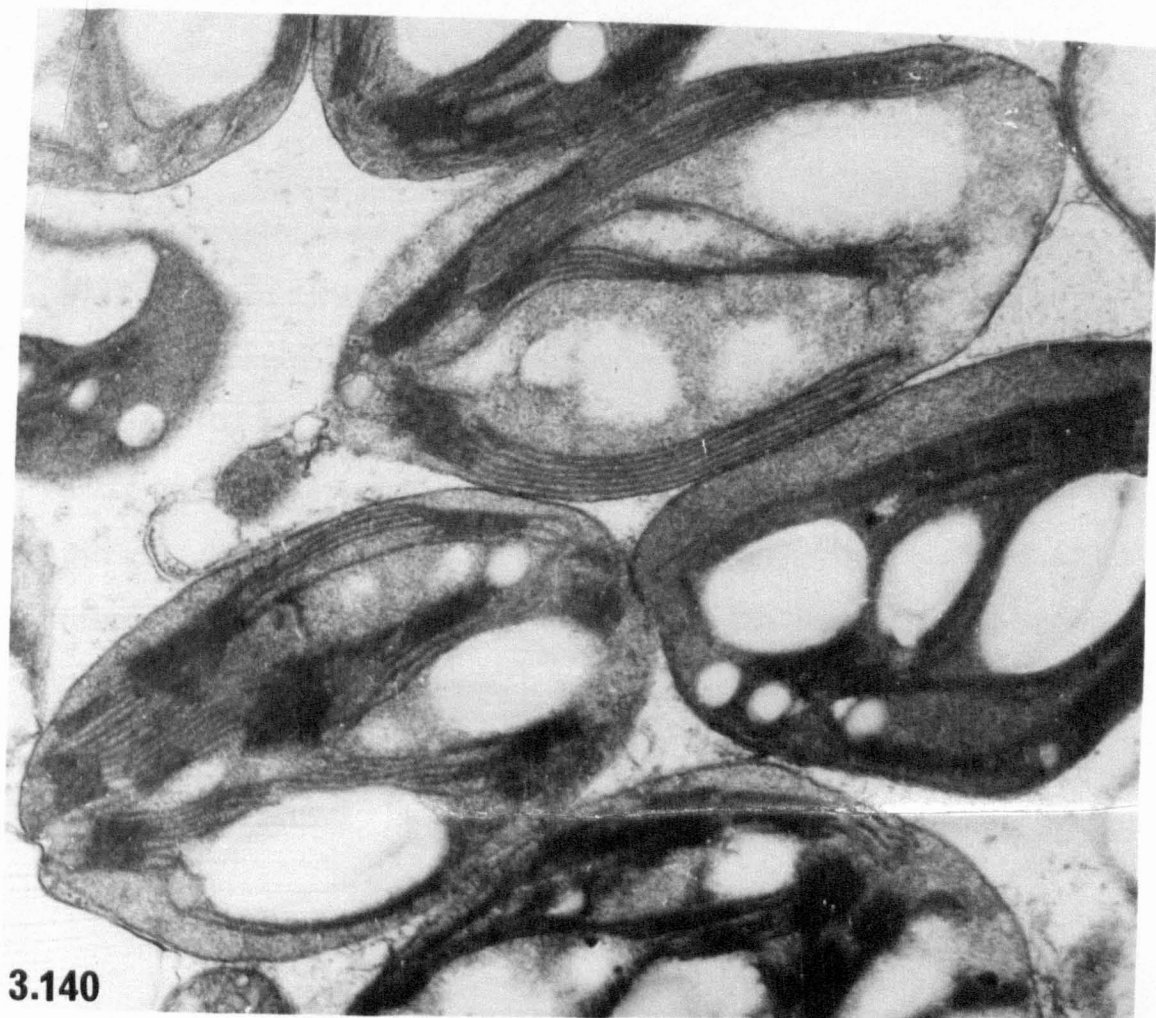


The culture of spontaneous fusion bodies

chloroplast structure

Figure 3.140 Chloroplasts in a fusion body culture for 9 days. They are closely packed, rounded and have a reduced lamellar content. Starch storage is considerable. x 22,500

Figure 3.141 As above(cultured for 13 days). The lamellae are rather more irregularly arranged. The tonoplast (t) has detached from the cytoplasm in one region. x 10,000



and cosomes. (see below). (Figs. 3.144 and 3.152).

d. Cosomes

Large cytoplasmic vesicles identified as cosomes (Willison 1973) are seen in fusion bodies only, in the first day of culture. They are invariably located near nuclei often only separated by 40 nm to 100 nm . (Figs. 3.142 to 3.144). The outline is clear, marked by membrane, and varying in shape from irregular to spherical. At high magnification the cosome is seen to contain fragments of membrane (Fig. 3.145). Staining with phospho-tungstic acid-chromic acid to identify plasmalemma, stained the outer protoplast membrane specifically, if patchily. (Fig. 3.146). The contents of the cosome also stained although the limiting membrane did not. (Fig. 3.147).

Some cosomes appeared to fuse together forming large irregular bodies (Fig. 3.148) which, over the next 9 days became surrounded by a thick, irregular, somewhat laminated layer of material. (Figs. 3.149 to 3.151). The matrix of the cosome shows some synthetic activity also. This degree of development was invariably associated with cytoplasmic breakdown and poor outer wall regeneration.

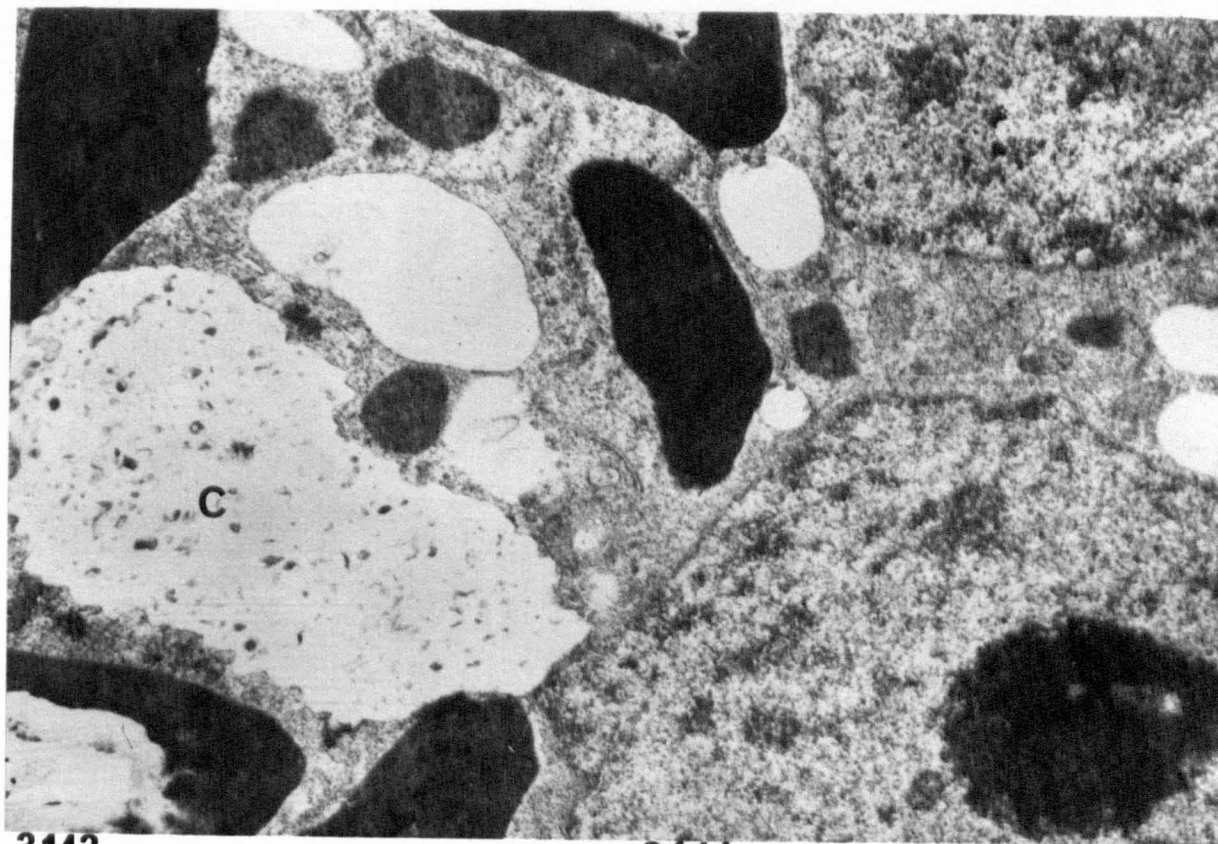
A contrasting pattern of development, usually occurring in the smaller fusion bodies, involved activity in the cosome matrix, (Fig. 3.152 and 3.154), but no thick outer layer was formed. At 4 days parallel e.r. and golgi like bodies accumulate near the cosome, (Figs. 3.152 and 3.153) and its outer membrane becomes indistinct and undulate. Between 6 and 12 days, cosomes were observed lying outside the cytoplasm often separated from it by a thick laminated wall (Figs. 3.154 and 3.188), with only a thin

The culture of spontaneous fusion bodies

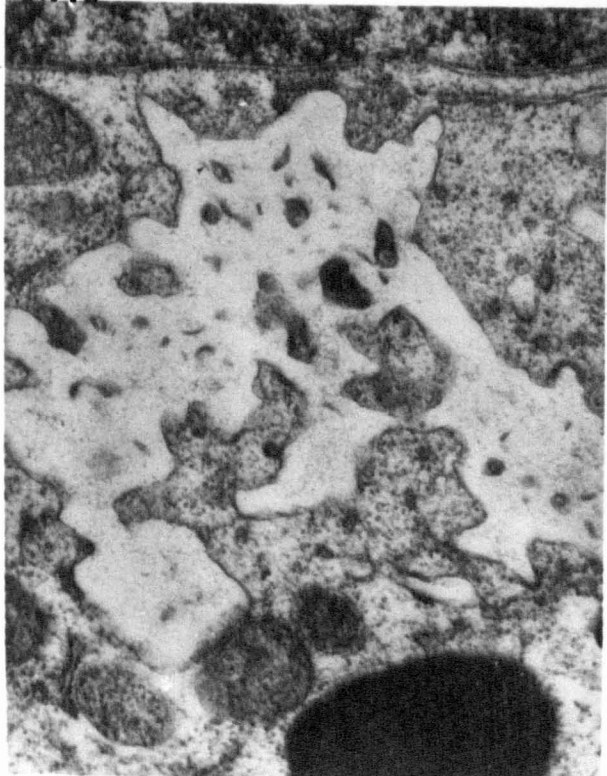
cosomes

- Figure 3.142 A cosome (c) lying near one of two nuclei in a
spontaneous fusion body. x 17,250
- Figure 3.143 An irregularly shaped cosome(possibly a tangential
cut through the periphery of a larger cosome),
lying near a nucleus.(Top of figure.) x 30,000
- Figure 3.144 As Figure 3.142. x 30,000

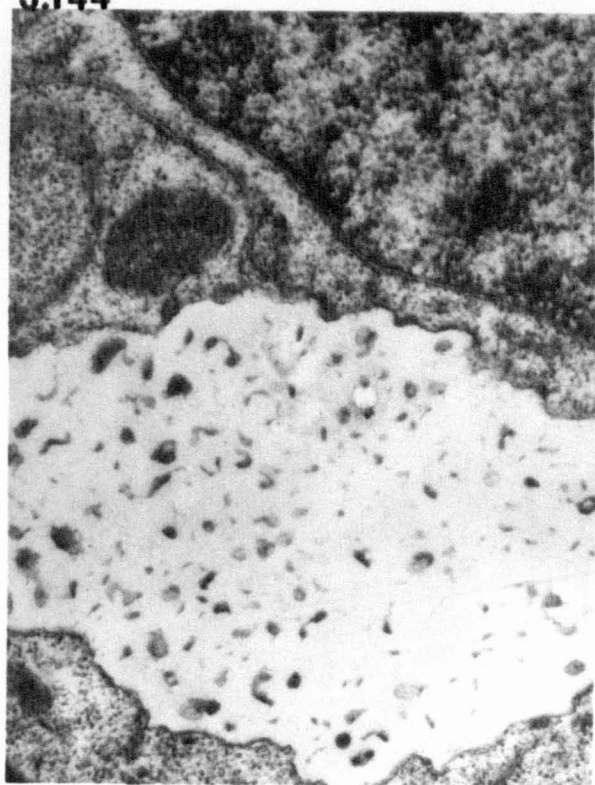
3.142



3.143



3.144



The culture of spontaneous fusion bodies

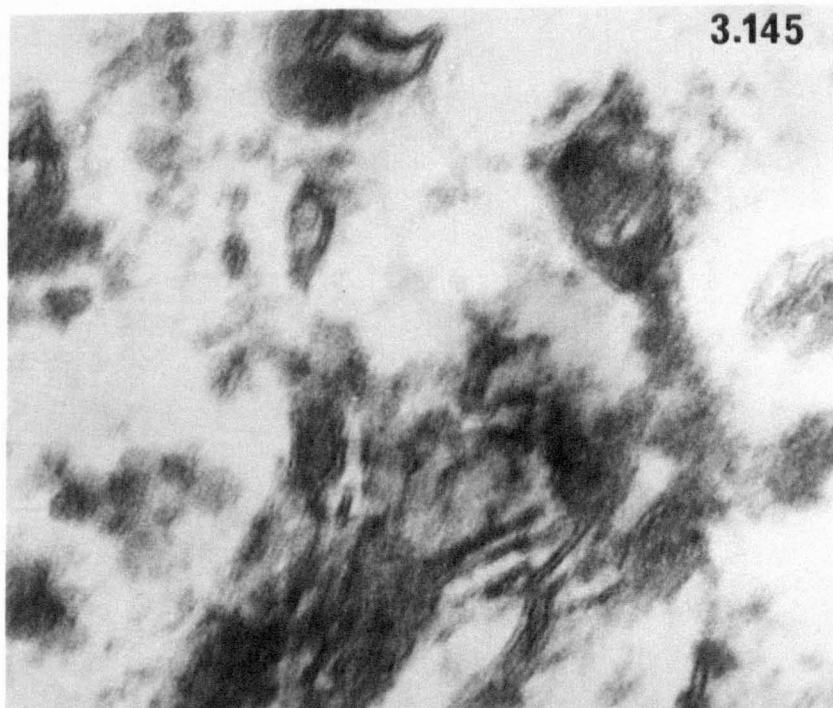
cosomes

- Figure 3.145 Fragments of bilayered membrane lying within
a cosome. x 95,000
- Figure 3.146 Patchy staining of the protoplast plasmalemma with
phosphotungstic acid-chromic acid stain. x 25,000
- Figure 3.147 A cosome stained as above. The contents stain
darkly although the limiting membrane does not.
x 45,000

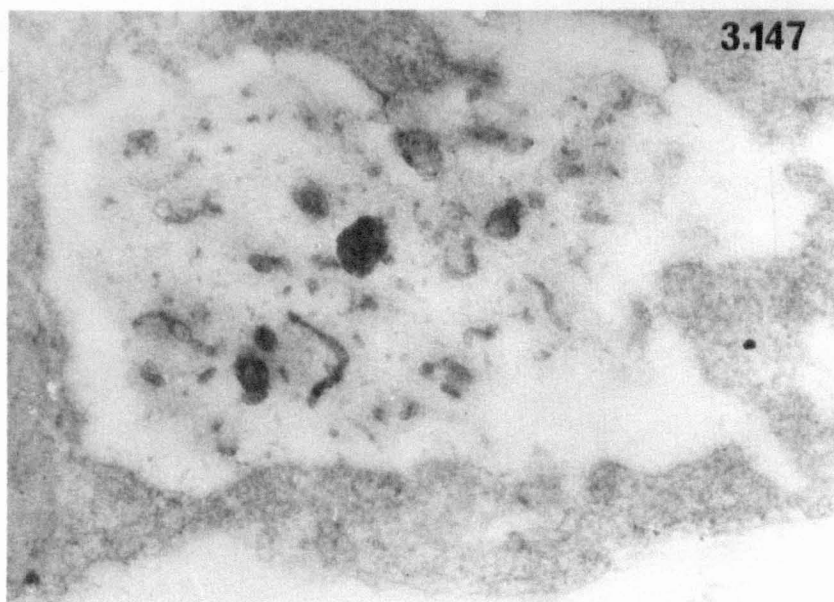
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3.145



3.147

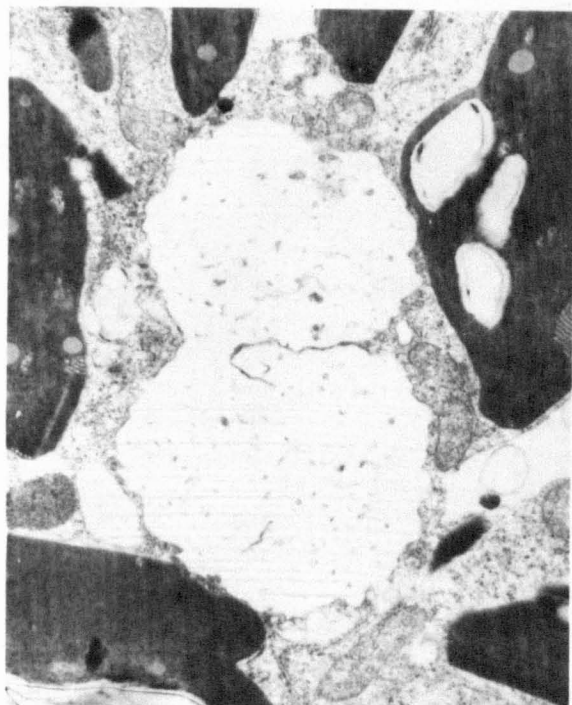


The culture of spontaneous fusion bodies

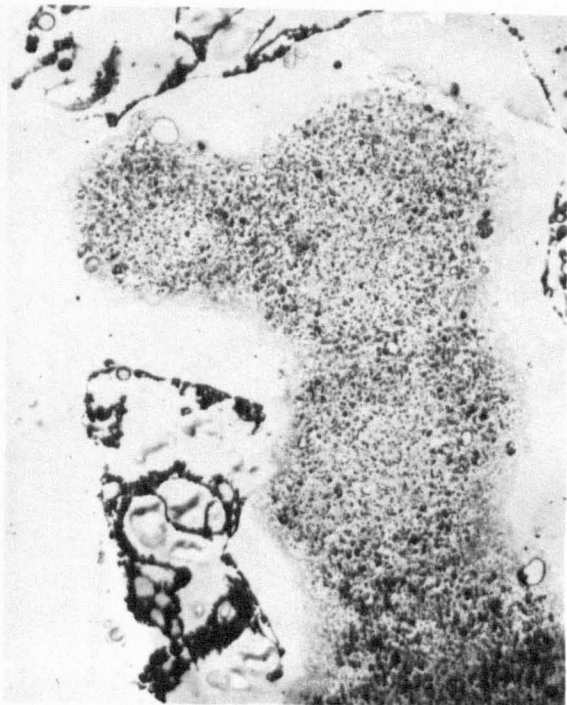
cosomes

- Figure 3.148 Two cosomes apparently fusing together after
one day in culture. x 12,000
- Figure 3.149 Several fused cosomes lying in degenerating cytoplasm.
A layer of newly synthesized material surrounds the
cosomes.(Fusion bodies cultured for 6 days)
x. 7,500
- Figures 3.150 and 3.151 The cosomes are surrounded by an
irregular fibrous layer. Spurs of the fibrous
material extend into the cytoplasm.(Fusion bodies
cultured for 12 days). Figure 3.150 x 8,000
Figure 3.151 x 12,000

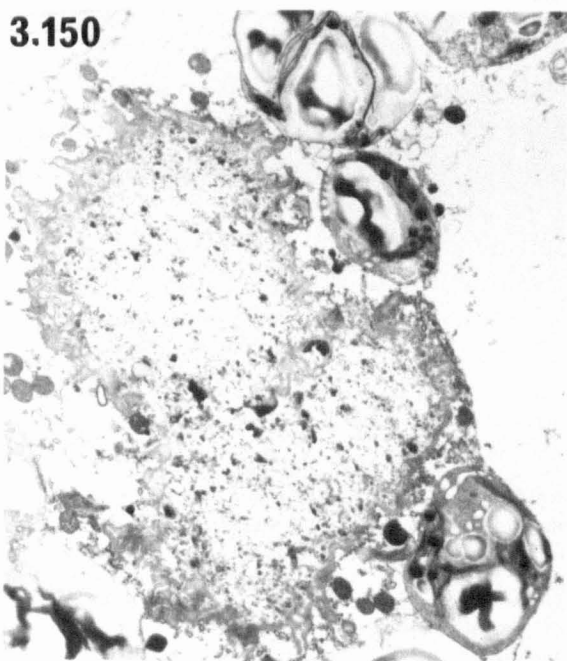
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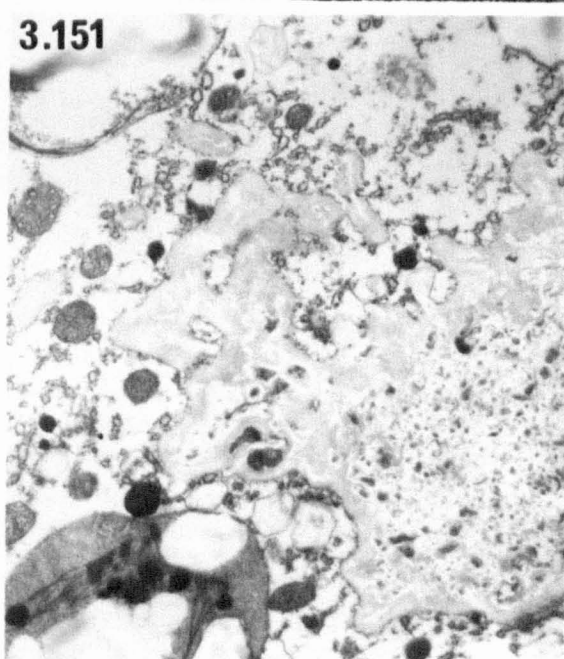
3.149



3.150



3.151

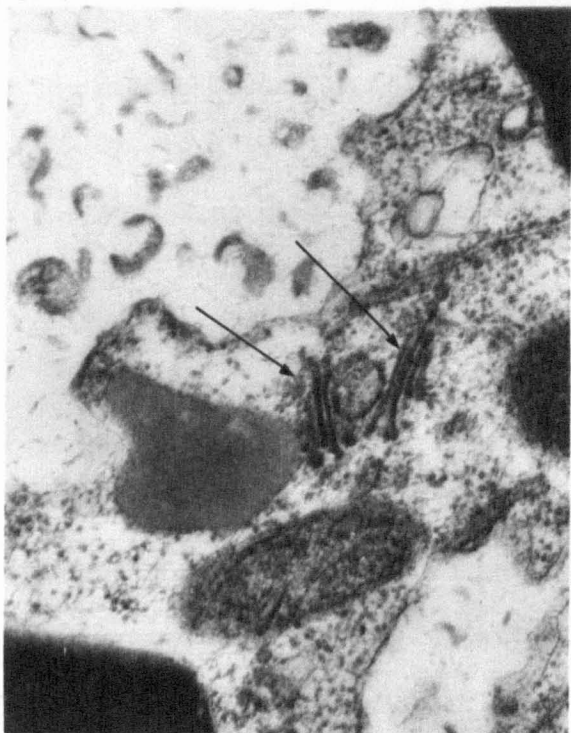


The culture of spontaneous fusion bodies

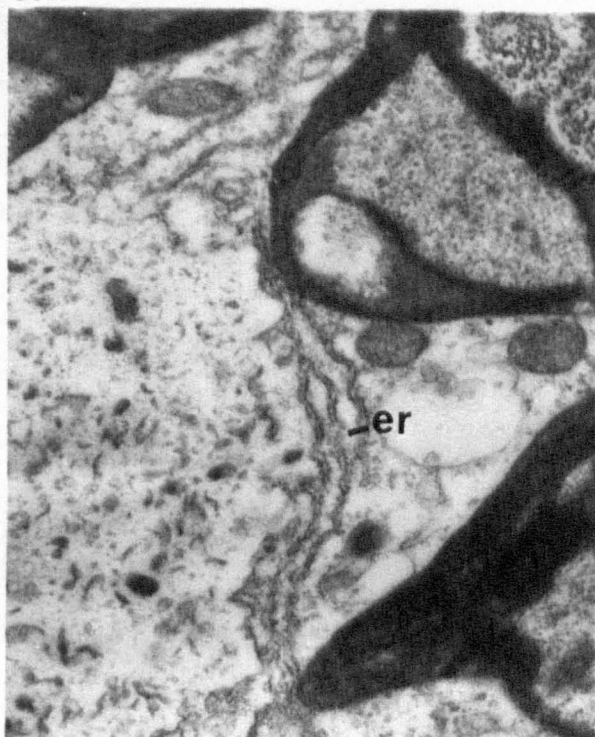
cosomes

- Figure 3.152 Parallel e.r. membranes lie near the cosome. The limiting membrane of the cosome is indistinct.
x 30,000
- Figure 3.153 Two golgi like structures lie near the cosome.
(Figs. 3.152 and 3.153 show material cultured for four days.)
x 40,000
- Figure 3.154 Two cosomes lying outside a fusion body but within a cleft lined by a thick laminated wall.(See Fig. 3.185). The cosome contents are bound together by a fibrous matrix.(c.f. Fig. 3.142).(Fusion body cultured for 13 days.) A very thin
x 8,000
fragmented wall lies on the outer side of the cosomes.(arrowed)

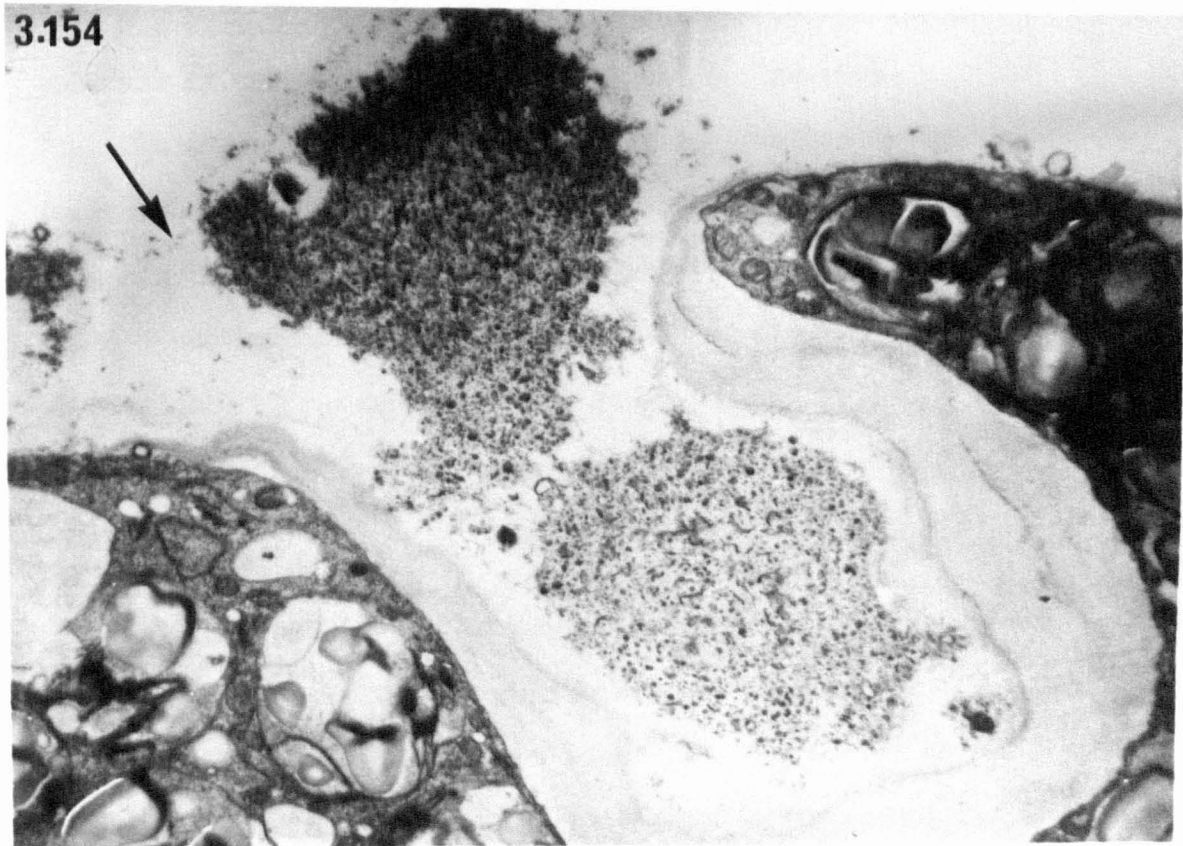
3.153



3.152



3.154



fragmented wall, if any, outermost. (Fig. 3.154 arrows). A distinct darkening of the outer side of the cosome and occasionally the entire cosome is observed. (Fig. 3.188).

By 12 days in culture, the only cosomes remaining in healthy fusion bodies were small and apparently inert. (Fig. 3.187h).

e. Tonoplast, vacuole and cytoplasmic vesicles.

During rounding off, the vacuolar system of the fusion body is reduced to a single large vacuole and some small vesicles. After 4 days in culture (6 for uninucleate protoplasts) dark staining material appears in the vesicles, (Figs. 3.170 and 3.171) and in the vacuole, (Fig. 3.172), attached usually to the tonoplast. Some larger globules of material have a fragmented core (Fig. 3.173), this may be a fixation artifact. Granular material was occasionally observed in vesicles, (Figs. 3.185 b and c, arrows).

The tonoplast fixes well limiting and containing the cytoplasm. However, a number of observations were made of the tonoplast detaching from the cytoplasm without breaking. (Fig. 3.141). This would appear artifactual but corresponds to frequent light microscopic observations of reversible tonoplast changes in cultured tomato fruit protoplasts. (J. H. M. Willison, personal communication).

f. Nuclei

As described in 3.3.4 there is a high degree of nuclear association in rounding-off fusion bodies. During the first day in culture the associations become clarified, the nuclei almost invariably forming pairs. Only two groups of three and two of four nuclei were observed in the entire study. Figure 3.155 shows twelve nuclei in one plane with three definite pairs present. The nuclear

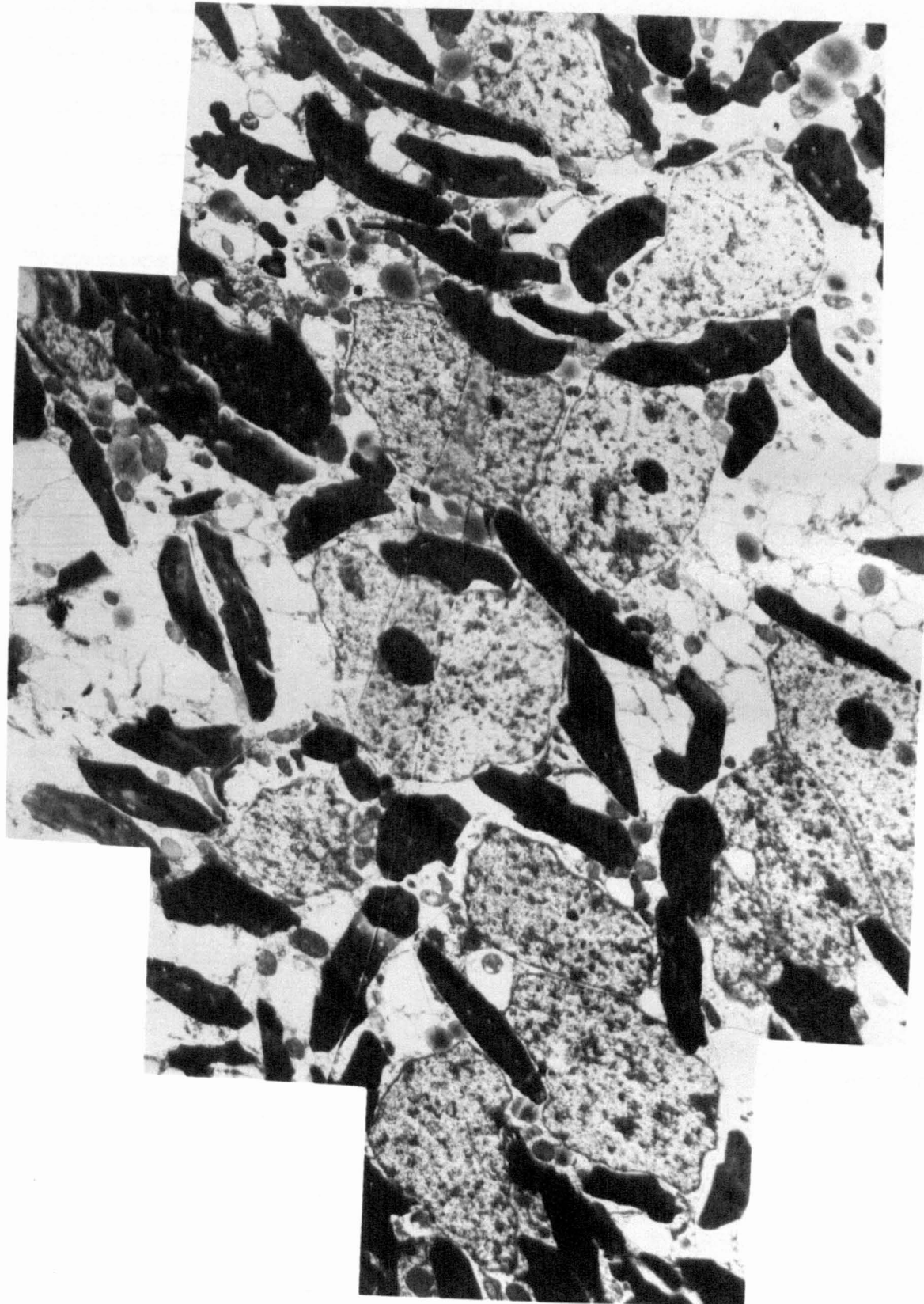
The culture of spontaneous fusion bodies

Nuclei

Figure 3.155 Montage electron micrograph showing twelve nuclei in a tobacco mesophyll spontaneous fusion body cultured for 1 day. Six of the nuclei are paired. The internuclear spacing is approximately 130nm.

x 6,000

3.155



membranes run more or less parallel at a spacing of 100nm to 150nm .

Thinner sections at higher magnification reveal more information about the nuclear associations. Initially e.r. membranes lie between the nuclei, (Fig. 3.156) some attached to one, (Fig. 3.157) or both nuclei (Fig. 3.158a). Usually only the outer nuclear membrane is involved but one observation was made of a junction apparently involving both membranes. (Fig. 3.159). In time only short membrane connexions are observed. (Figs. 3.158b, 3.160 to 3.162). The latter Figure shows a membrane bridge with a spacing virtually identical to that separating the two nuclear membranes. ($\approx 25\text{nm}$)

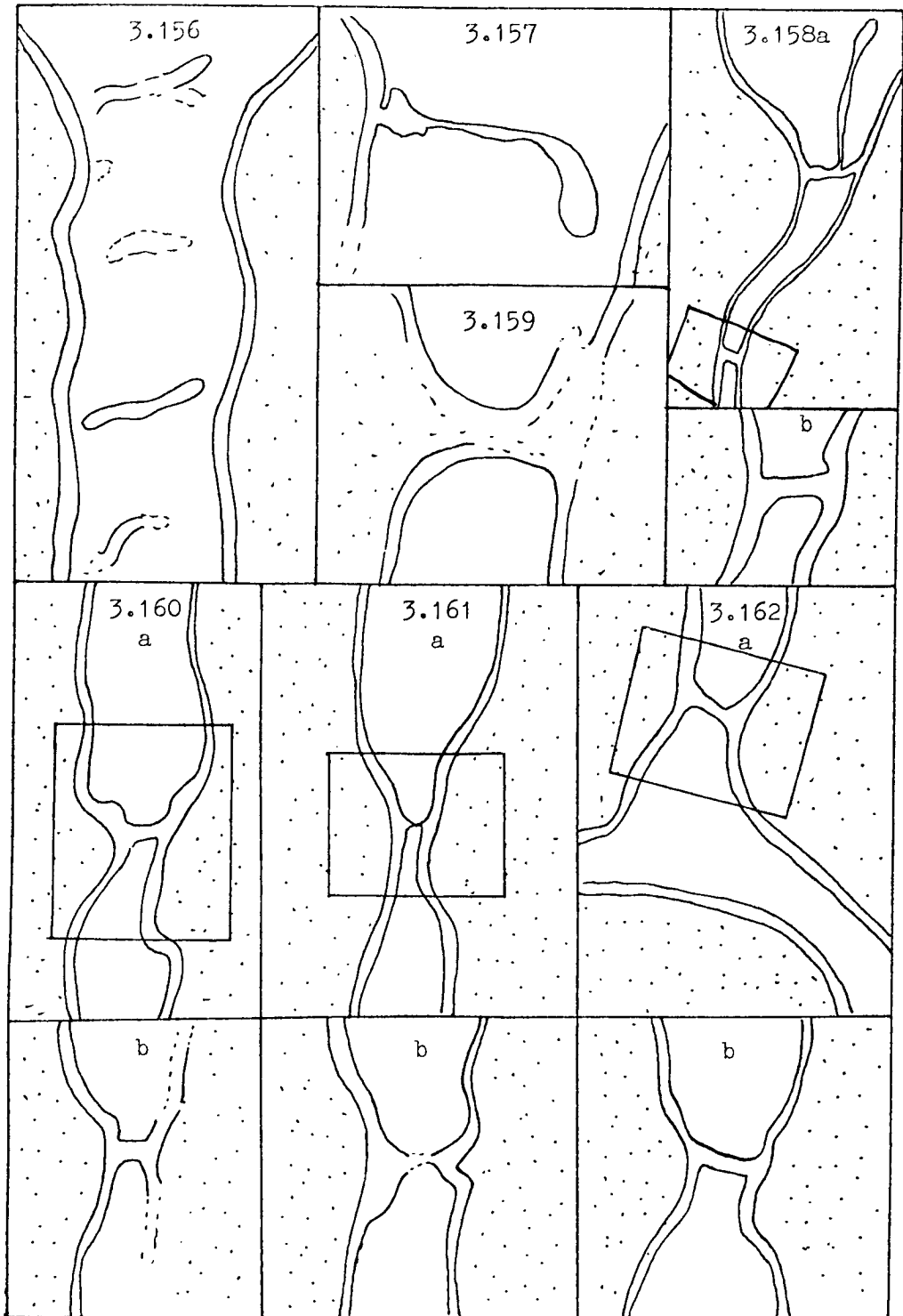
By the second day in culture most of the nuclei are dissociated and such a high degree of association was only observed again in some of the fusion bodies undergoing division. (Fig. 3.186).

g. Wall formation and cell division

Regeneration of the outer wall is a rapid process, cellulose fibrils being visible in surface replicas of protoplasts at a very early stage. (B. Grout, personal communication). In thin section a considerable amount of material may be necessary for visualization of the wall. A near perpendicularly sectioned protoplast may reveal little (Fig. 3.163) although a glancing tangential section indicates the presence of much fibrillar material. (Fig. 3.164). Both these figures show an organization of microtubules and ramifying e.r. beneath the plasmalemma.

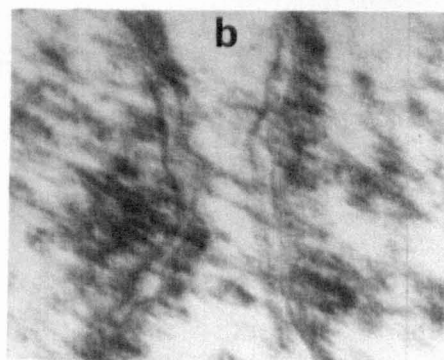
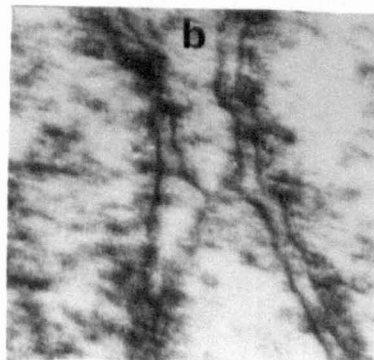
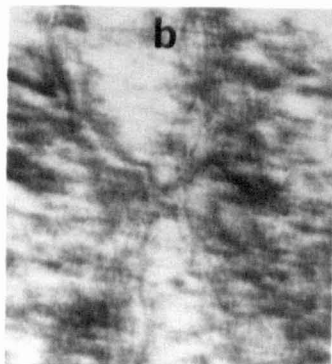
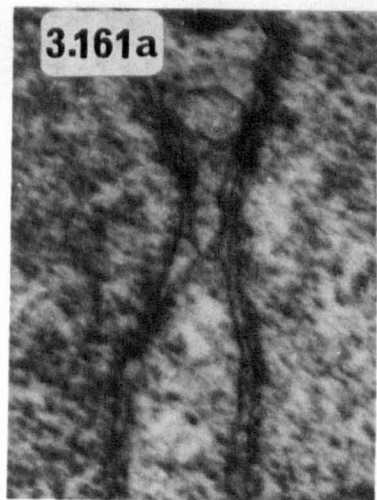
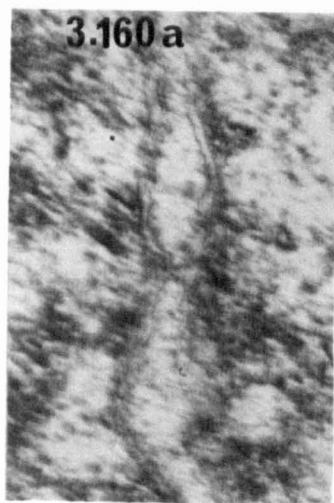
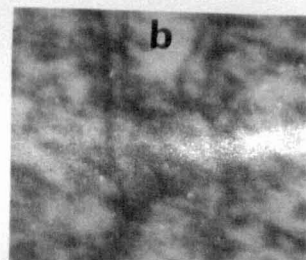
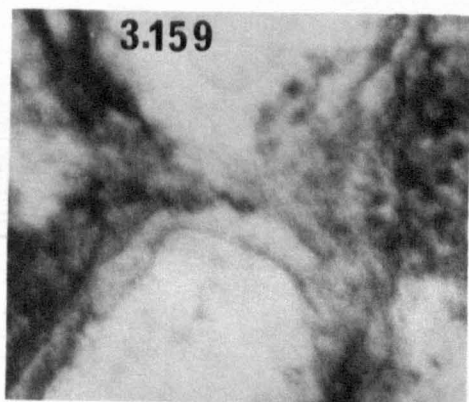
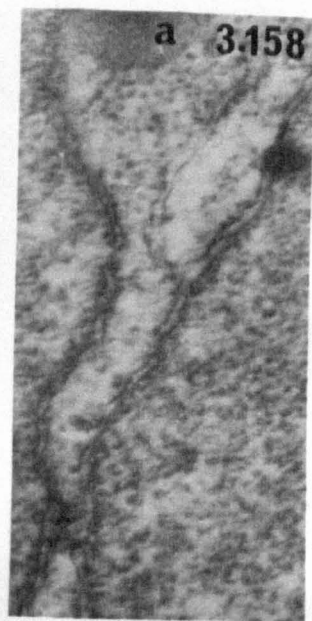
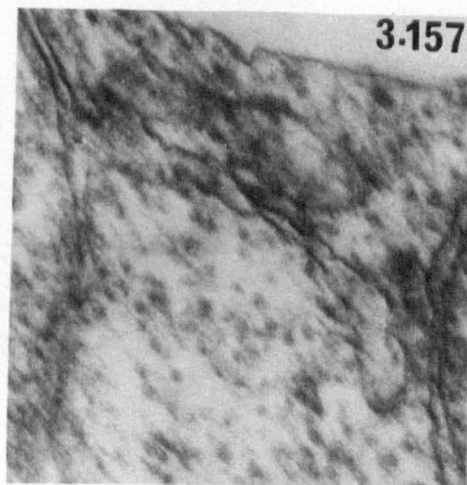
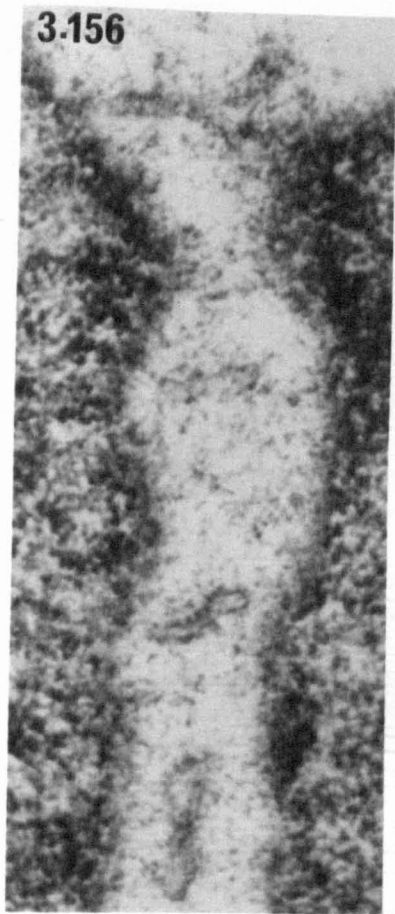
The attachment of a cosome to a protoplast (fusion body) suggests the synthesis of some wall material although none may be visible. (Figs. 3.165a and b). (Figures 3.163 to 3.165 are from 4 day cultures).

The rate of wall regeneration is neither constant with time nor even



(Enlarged section are boxed in the lower power figures)

- Figure 3.156 E.r. lying between, and connecting nuclei. x 42,000
 3.157 " x 90,000
 3.158a " x 55,000
 b Short connexion of outer nuclear membranes. x100,000
 3.159 Connexion, possibly formed from outer and
 inner nuclear membranes. x 90,000
 3.160 to 3.162a Short connexion of outer nuclear membranes. x 60,000
 b " x120,000



The culture of spontaneous fusion bodies

wall formation

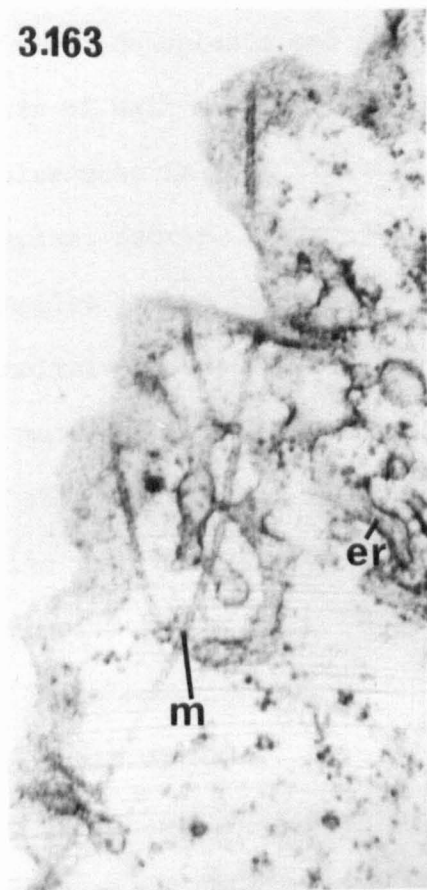
Figure 3.163 A near perpendicular section through a protoplast cultured for 4 days. E.r. and microtubules (m) can be seen beneath the plasmalemma, but no wall material is evident. x 45,000

Figure 3.164 A glancing tangential section of a similarly aged protoplast, which is regenerating a wall. Fibrillar material lies on the outside. Within the cytoplasm, e.r. and microtubules (m) can be seen. x 30,000

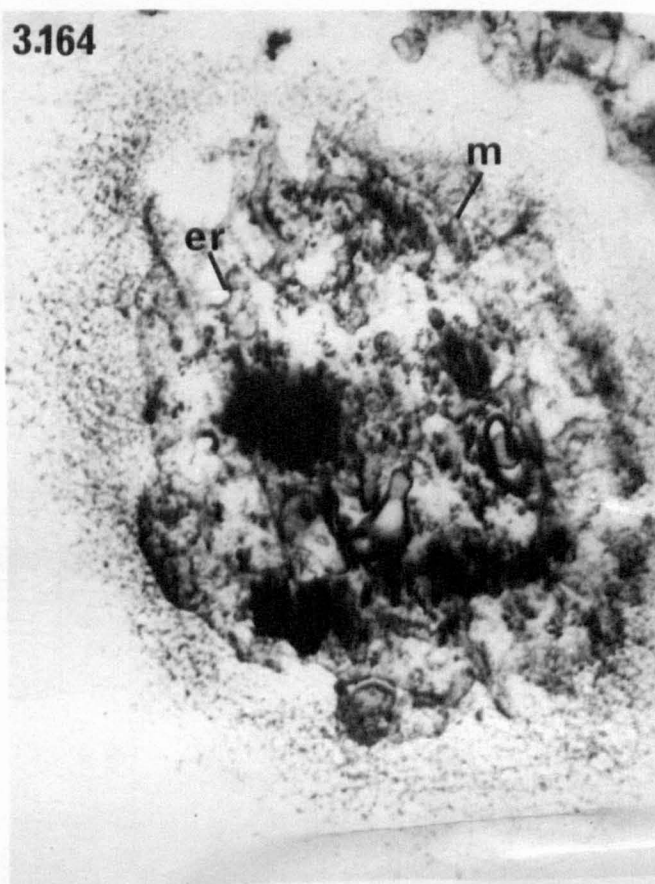
Figures 3.165a and b A spontaneous fusion body, cultured for four days. An expelled cosome (c) lies within a shallow depression in the fusion body. No wall material is evident, however. Figure 3.165a x 1,750

Figure 3.165b x 7,000

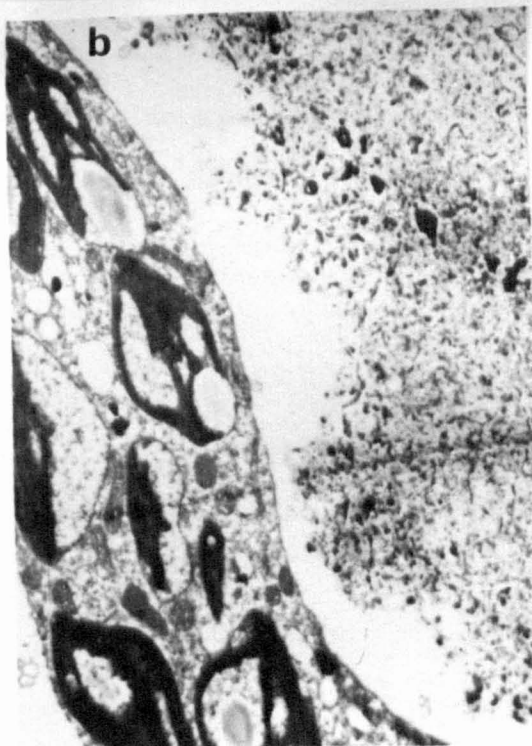
3.163



3.164



3.165a



over the entire protoplast surface, but by 12 days in culture, all surviving protoplasts and fusion bodies had regenerated appreciable amounts of wall material. Outside the fibrillar wall, dense black globular material was frequently observed, often continuous with dead protoplast debris. (Fig. 3.166). Close inspection identified cytoplasmic organelles in one large accumulation of material. (Fig. 3.167). Where plasmolysis and secondary wall formation had occurred, no second layer of this material was observed. (Fig. 3.168). Irregularities in this layer were observed. (Fig. 3.169). Fusion bodies tended to form the layer earlier than uninucleate protoplasts although not all had an appreciable amount of material.

The classification of internal walls proves difficult. Some are clearly non-mitotic. (Figs. 3.174 a and b). Here a tangential wall cuts off a peripheral region of non-chloroplast containing cytoplasm. Partial subdivision of this appears to be occurring. Debris suggestive of a similar earlier event is seen. (Fig. 3.174a, arrows).

The structure in Figures 3.175a and b has an equal division with (probably) later subdivisions. The upper region of the body is an isolated area of vacuolated (see Fig. 3.175b, arrow) cytoplasm, rich in mitochondria. The two lower regions have sparse cytoplasm, much vacuolar globular material and the dense cytoplasm of the central section is dividing into many small subunits. Only one clear chloroplast is evident. (Fig. 3.175b, double arrow). The body shown in Figure 3.176 has an incomplete division, partially separating a peripheral region. The wall appears to be growing centripetally. Here, and in Figure 3.175b (broken arrow) walls cutting off triangular areas are seen.

In Figures 3.177a and b, a protrusion of cytoplasm is subdivided by walls. Figure 3.178 in contrast, shows a two-armed wall lying unattached

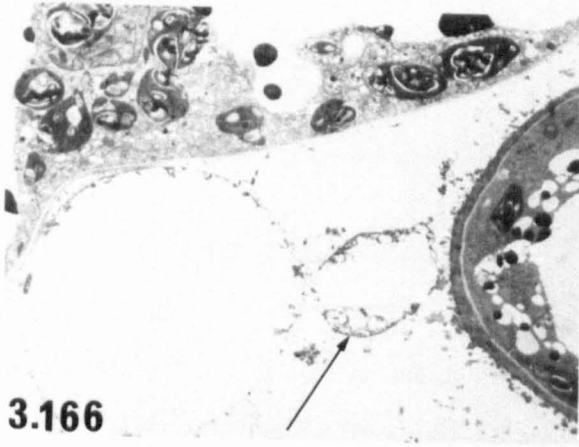
Osmiophilic substances produced during the culture
of tobacco mesophyll protoplasts and spontaneous
fusion bodies

Figures 3.166 to 3.169 ... externally located

Figures 3.170 to 3.173 ... internally located

- Figure 3.166 The protoplast to the right has an outer layer of dense material which is continuous with protoplast debris in the medium (arrow). x 1,750
- Figure 3.167 Details of the dense material. A structure resembling a degenerating chloroplast can be seen. x 52,000
- Figure 3.168 A plasmolysed protoplast has regenerated a second wall (arrow) within the originally regenerated wall. Little dense material lies outside the second wall. x 4,000
- Figure 3.169 An irregularity in the layer of dense material. x 20,000
- Figure 3.170 Small globules of dense material attached to the limiting membrane of cytoplasmic vesicles. x 22,000
- Figure 3.171 Larger accumulations of material. x 45,000
- Figure 3.172 A large globule attached to the tonoplast of the main vacuole. The deformation of the tonoplast may be a fixation artifact. x 50,000
- Figure 3.173 A large mass of material lying in the vacuole. Its core is somewhat fragmented, again possibly artifactual. x 10,000

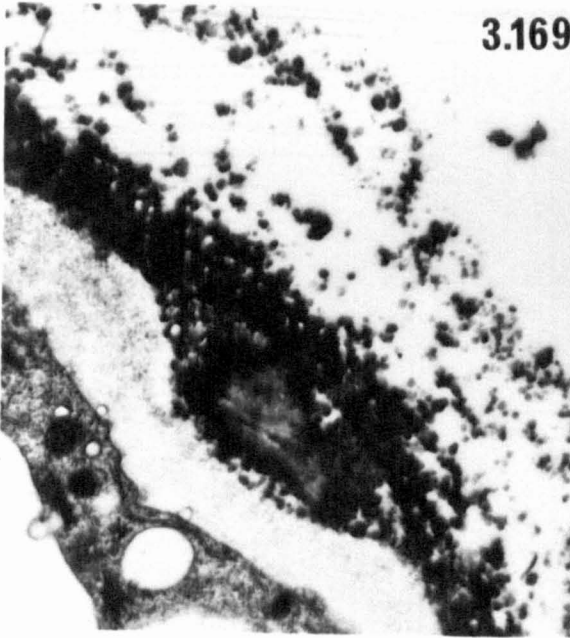
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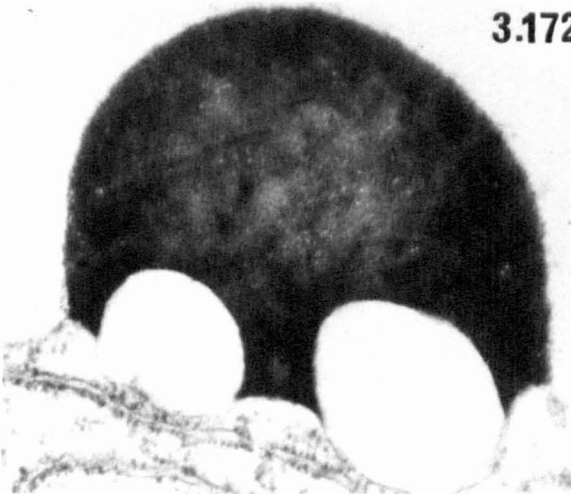
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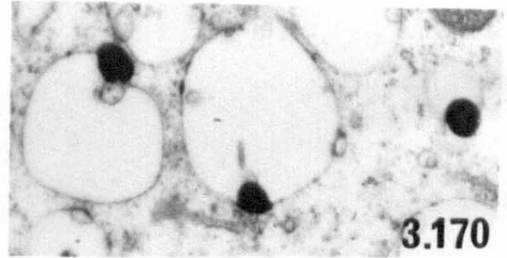
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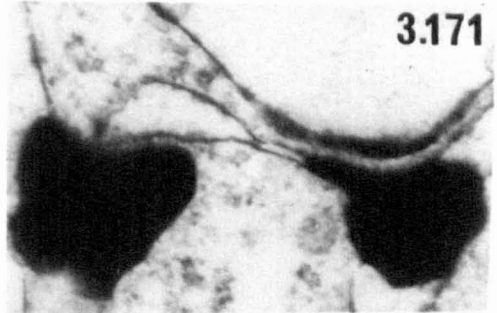
3.167



3.170



3.171



3.173



The culture of spontaneous fusion bodies

dividing wall formation

Figures 3.174 a and b (From adjacent sections) A peripheral region of cytoplasm is separated by a dividing wall. The partitioned cytoplasm is itself subdivided. An earlier division appears to have occurred. This section has however degenerated.(arrows). (Cultured for 10 days.) Figure 3.174 a x 1,250

Figure 3.174 b x 4,500

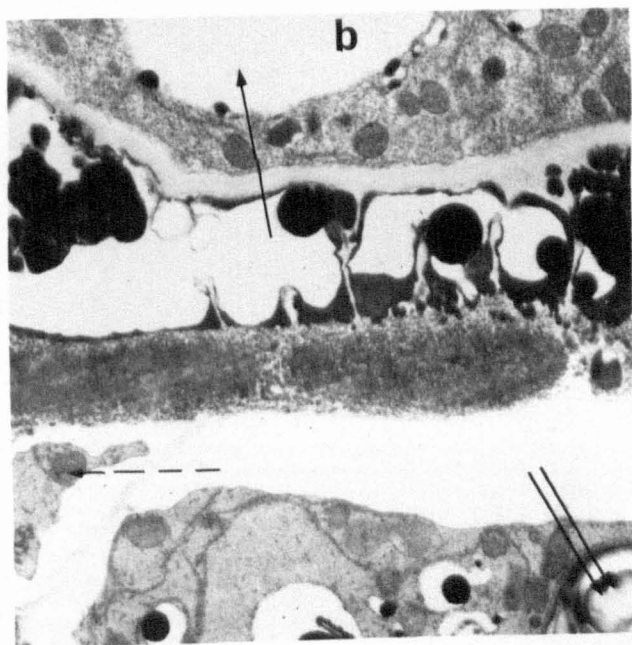
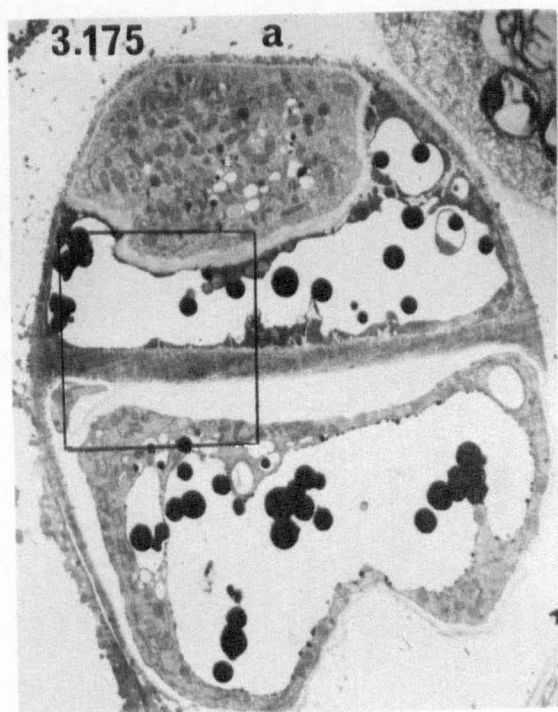
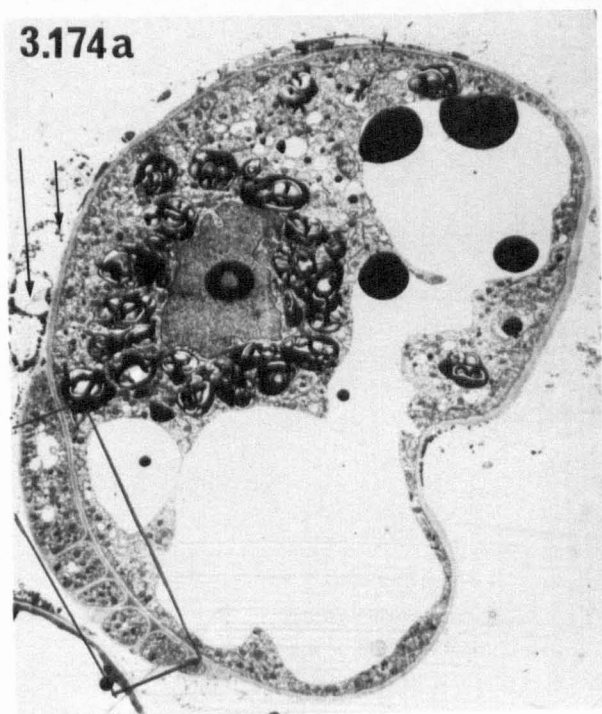
Figures 3.175 a and b (From adjacent sections) A subdivided protoplast. The upper(vacuolate,arrow) region is rich in mitochondria. The middle and lower sections contain a considerable amount of dense globular material. The middle section,which lacks any live cytoplasm, appears to be subdividing. To the left of The lower region, a triangular region of cytoplasm has been cut off.(Broken arrow) A chloroplast is indicated by double arrows.(Cultured for 12 days).

Figure 3.175 a x 4,250

Figure 3.175 b x 8,500

Figure 3.176 A degenerating protoplast; an incomplete, centripetally growing wall joins other subdividing walls to form triangular subdivisions.(Cultured for 10 days.)

x 2,500

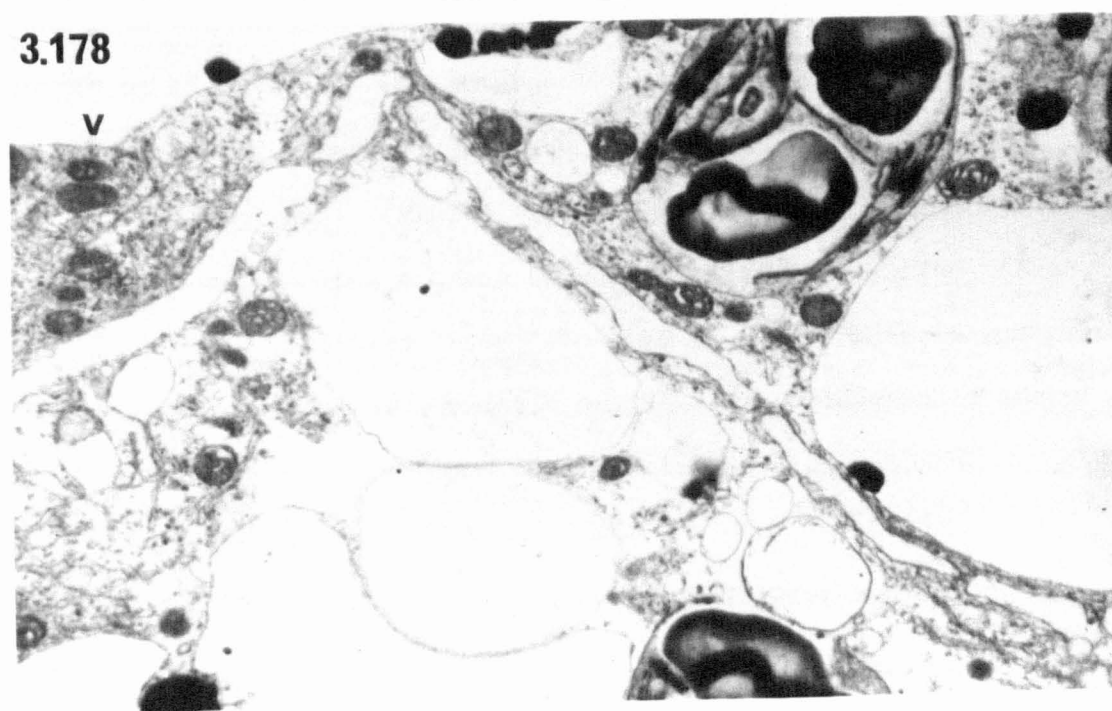
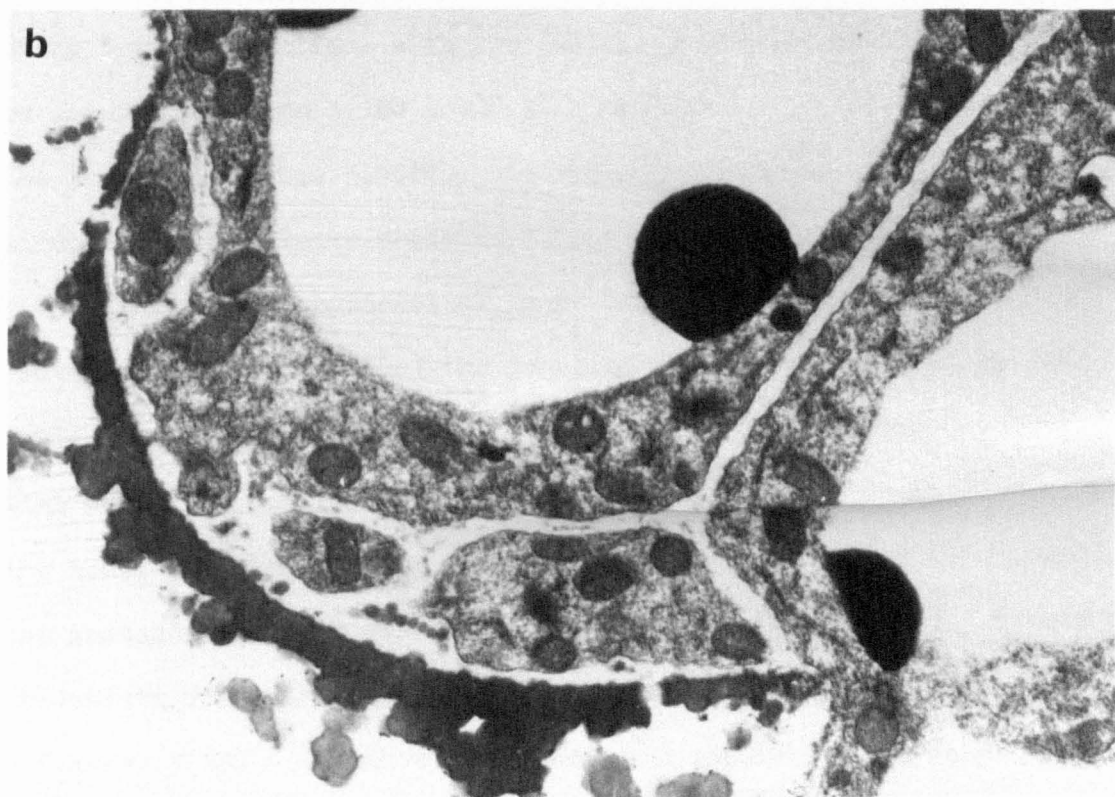
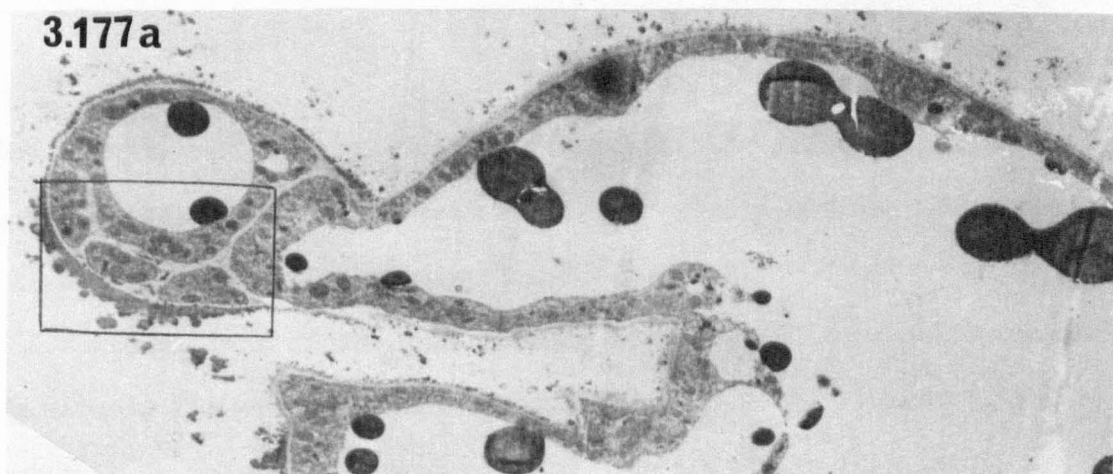


The culture of spontaneous fusion bodies
dividing wall formation

Figures 3.177 a and b (From adjacent sections) A cytoplasmic projection is subdivided by several internal walls. A considerable amount of densely staining material lies on the outside of the projection. The cytoplasm is chloroplast free in this region. (Cultured for 10 days; 10% sorbitol in medium) Figure 3.177 a x 2,500

Figure 3.177 b x 12,000

Figure 3.178 A two armed internal wall lies in the cytoplasm of the main region of a fusion body. The wall approaches the vacuole (v) but is not connected to the outer walls. (Cultured for 8 days.) x 20,000



in the cytoplasm. Internal, non mitotic, dividing walls were seen in both uninucleate protoplasts and spontaneous fusion bodies. None reacted positively to the pectin staining technique.

A few divisions of the type common to tissue culture cells undergoing mitosis (Roberts and Northcote, 1970) were seen, (Figs. 3.179a - c) but only in uninucleate protoplasts. The initially thin irregular wall thickens forming junctions with the (usually) thinner outer wall, of the types shown in Figures 3.180 a and b. Occasional subdivisions are seen at the junction. (Fig. 3.180d). In contrast, very few divisions in multinucleate protoplasts could be definitely associated with mitosis. They were invariably asymmetrical, some being at one end continuous with the outer wall, (Fig. 3.180c) the junction occasionally marked by the presence of a cosome (e.g. junction seen in Figure 3.180e.) The apparently growing end of the wall rarely made complete contact with the other side of the body, sometimes ending abruptly in the cytoplasm. (Fig. 3.181a). In one instance this almost touched a nucleus. (Fig. 3.181b). A very few plasmodesmata were observed. (Fig. 3.181c).

Figures 3.182 a - c show the apparent incomplete partitioning of a nucleus from the rest of the multinucleate fusion body. In these figures a protrusion of cytoplasm can be seen (arrows). These were common, particularly in low osmoticum cultures. They were usually small, containing few if any chloroplasts. (Fig. 3.183). Some, however, contained chloroplasts and nuclei, forming a chain of several nucleated units. (Figs. 3.184a to c). The latter was never observed in high osmoticum cultures.

Divisions, possibly of a mitotic origin, were occasionally seen in fusion bodies. Figures 3.185 a - c are serial sections showing a division incompletely separating a cytoplasmic from a vacuolated region. Both regions are nucleate. One end of the cross wall joins the outer wall,

CO

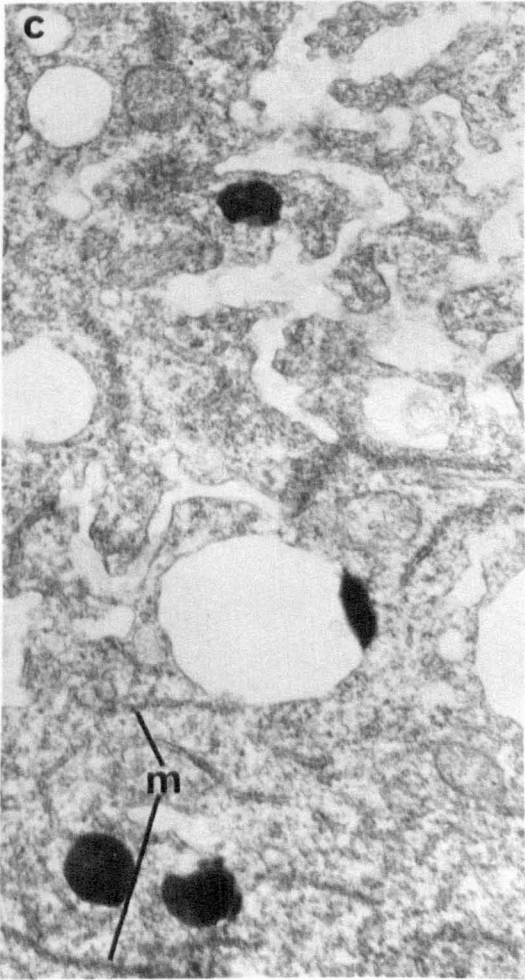
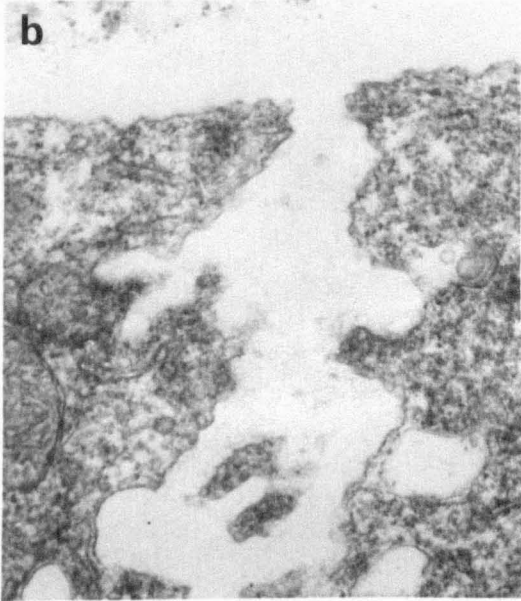
Mitotic division in a cultured tobacco
mesophyll protoplast

Figure 3.179 a Low power view of the dividing wall. Chloroplasts line one side of the wall, with mitochondria predominating on the other side. x 6,500

Figure 3.179 b A detail of the end of the wall attached to the outer wall. x 20,000

Figure 3.179 c A detail of the end of the wall free in the cytoplasm. The elements of the wall become highly irregular. Microtubules (m) are seen running more or less at right angles to the plane of the wall. x 12,000

3.179



Aspects of division wall formation in cultured tobacco
mesophyll spontaneous fusion bodies

Figures 3.180 a and b Junctions of cross walls and outer walls resembling those observed in dividing uninucleate protoplasts.

Figure 3.180 a x 20,000

Figure 3.180 b x 15,000

Figure 3.180 c The cross wall is continuous with the outer wall.
x 15,000

Figure 3.180 d Subdivision of the cytoplasm at the junction.
x 12,000.

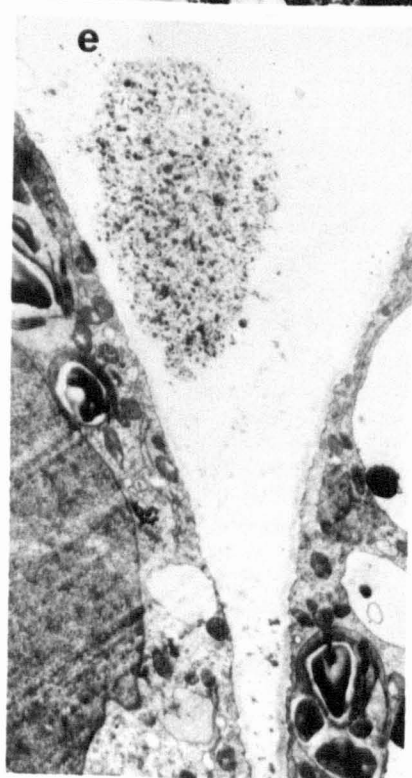
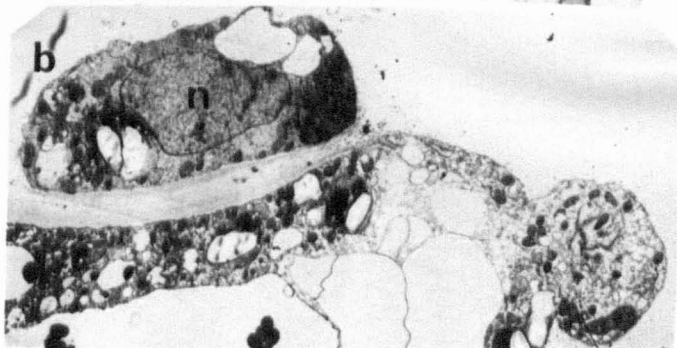
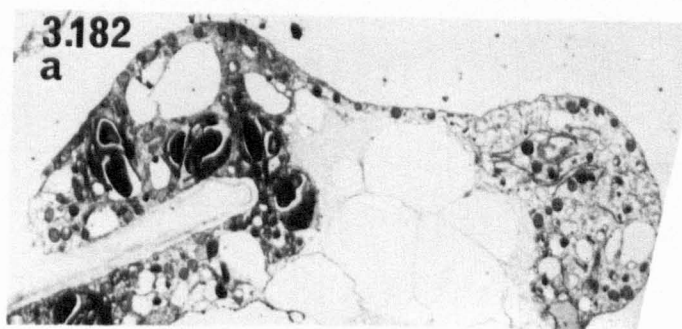
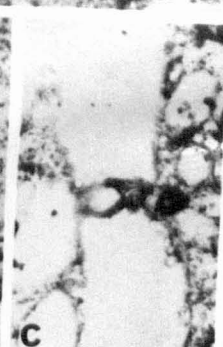
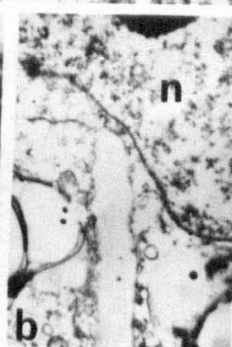
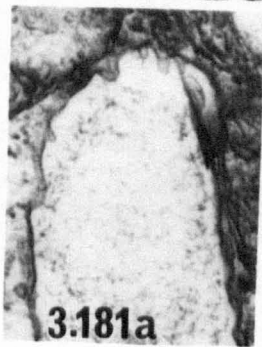
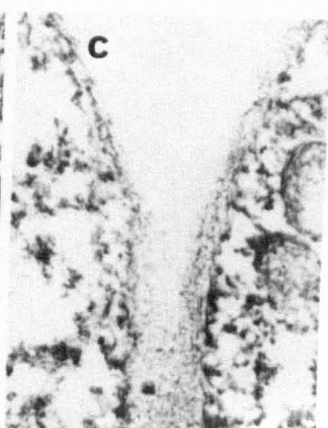
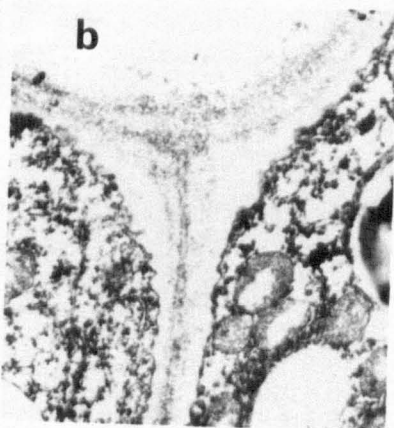
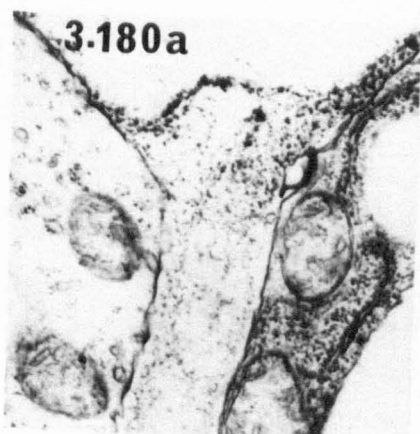
Figure 3.180 e A cosome lies in the cleft between the two halves of the dividing wall.
x 12,000

Figure 3.181 a The apparently growing end of a dividing wall terminates in the cytoplasm.
x 45,000

Figure 3.181 b As above, the wall ends near a nucleus.(n).
x 20,000

Figure 3.181 c One of the few plasmodesmata seen in the dividing walls.
x 15,000

Figures 3.182 a to c Serial sections through a spontaneous fusion body; a nucleus is incompletely partitioned from the rest of the cytoplasm. To the right, a chloroplast-free protrusion is seen. (Cultured for 10 days; 13% sorbitol in medium.)
x 3,500

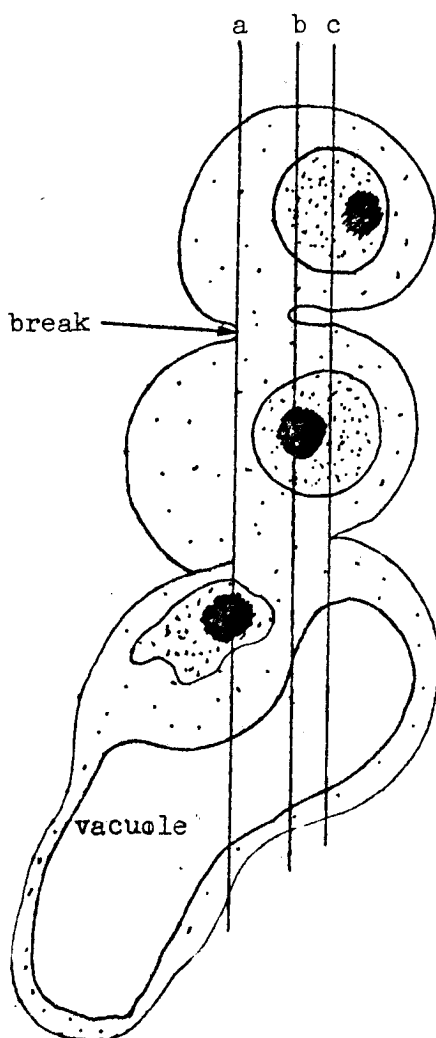


The culture of spontaneous fusion bodies, protrusion formation

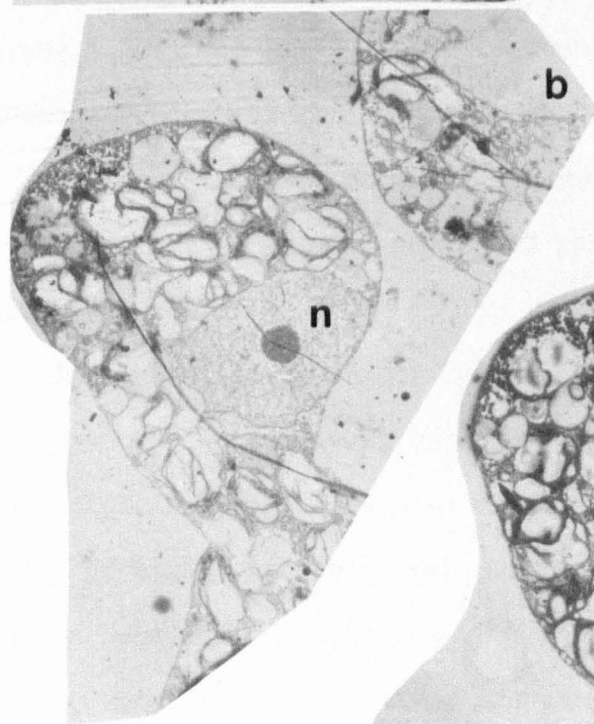
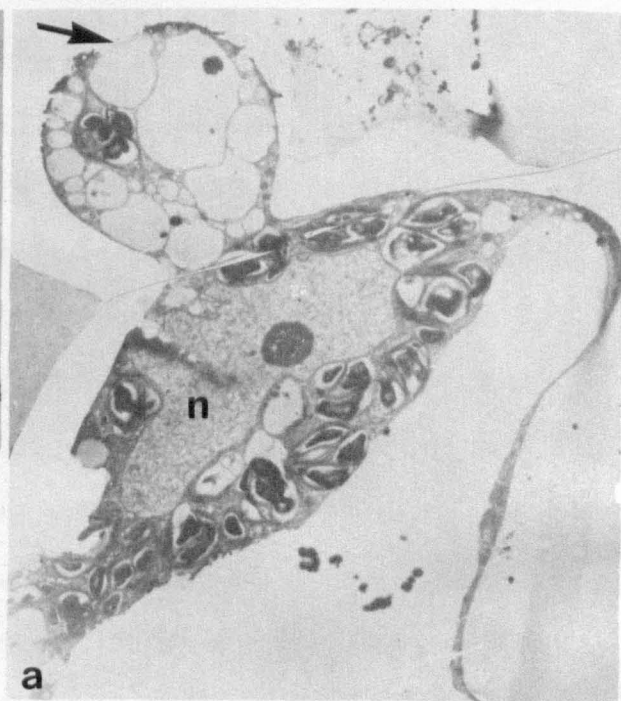
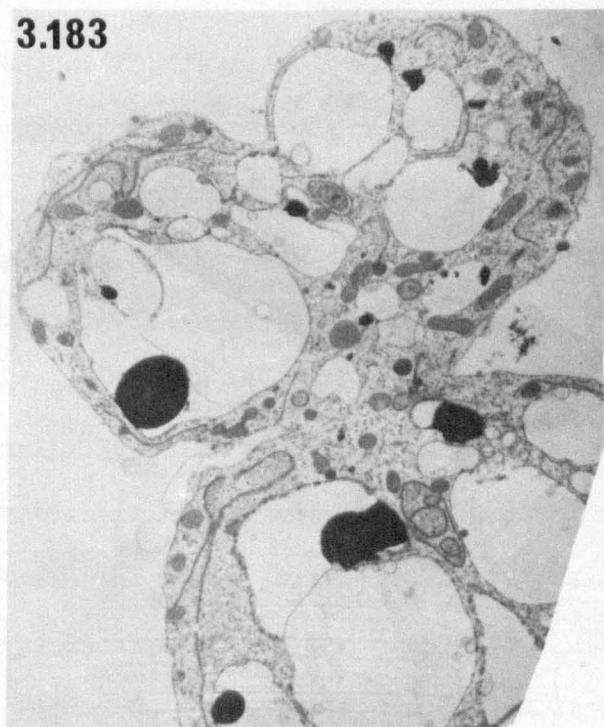
Figure 3.183 Two chloroplast free protrusions form from the main mass of cytoplasm. x 4,500

Figure 3.184 a to c Serial sections through a spontaneous fusion body which has formed several nucleated protrusions. In Figure 3.184a the embedding medium has broken away from the fusion body, separating a region of cytoplasm from the main body.(arrow). A nucleus (n) lies in the lower part of the body. In Figures 3.184 b and c two nuclei are seen in a protrusion from the fusion body and in a nearby sphere of cytoplasm. The diagram below indicates the suggested planes of the sections. (Cultured for 12 days; 10% sorbitol in medium)

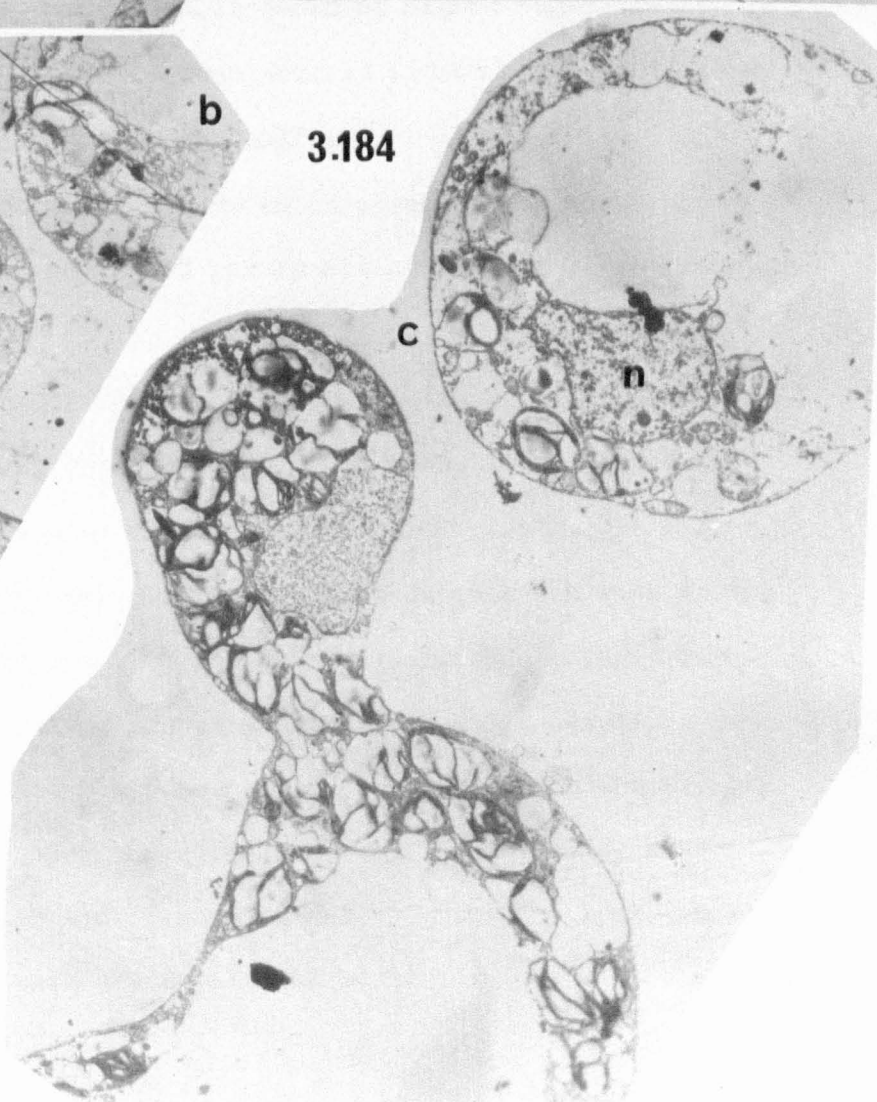
x 4.000



3.183



3.184



(Fig. 3.185a*) whilst the other ends in the cytoplasm. (Fig. 3.185d).

An apparent second wall is forming in a thickened region. (Fig. 3.185e).

A more complex situation is shown in the four serial sections of Figures 3.186 a to d. A tripartite wall isolating two closely associated nuclei is followed by a simple dividing wall running obliquely through the body. Some five nuclei are evident. The main cross wall has a very complex junction with the outer wall. (Figs. 3.187 a to g). Where the junction is incomplete, a dense, possibly membrane bound, structure lies between the walls (Fig. 3.187 e to g), and an undulating new wall runs perpendicularly from the cross-wall. (Fig. 3.187g, arrow). The junction of the three walls in Figure 3.186a is shown at higher magnification in Figure 3.187h. A small cosome is seen locally (inset) and there is an accumulation of mitochondria near the walls.

Walls of the type described above which possibly represent mitotic divisions reacted inconclusively to pectin staining, some slight reaction occurring.

The two dividing fusion bodies in Figures 3.185 to 3.187 are from 13 day cultures. A similarly aged fusion body is shown in Figure 3.188. Two nuclei, surrounded by many closely packed chloroplasts, lie near a region of highly irregular wall formation. Two cosomes lie outside the body (arrows), in association with an enormous wall thickening. Two others, very densely staining, are attached to the wall, indenting into the fusion body. Laminations are seen in the wall, (double arrows) and a wall appears to be crossing the cytoplasm from the thickened region. Sections taken from either side (insets a and b) show that both the wall thickening and internal wall are localised.

Division in a cultured spontaneous fusion body.(Cultured for 13 days.)

Figures 3.185 a to c Serial sections through the fusion body.

The two halves of the body are incompletely separated.
The division wall joins the outer wall at one end in
Figure 3.185 a (*) and at both ends in Figure 3.185 c.
Two nuclei on one side of the wall (Fig. 3.185 a) and
one or possibly two on the other side, can be seen.
Large cosomes are being extruded from the upper half
of the body.(See also Fig. 3.154.) x 1,250

Figure 3.185 d A detail from Figure 3.185 a. The thin outer wall
can be seen, with the cross wall(w) approaching it.

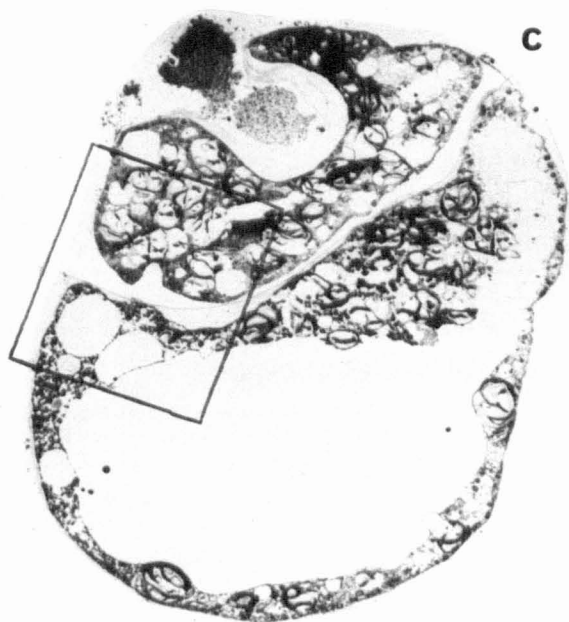
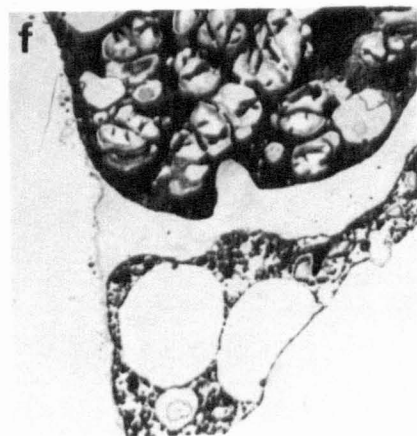
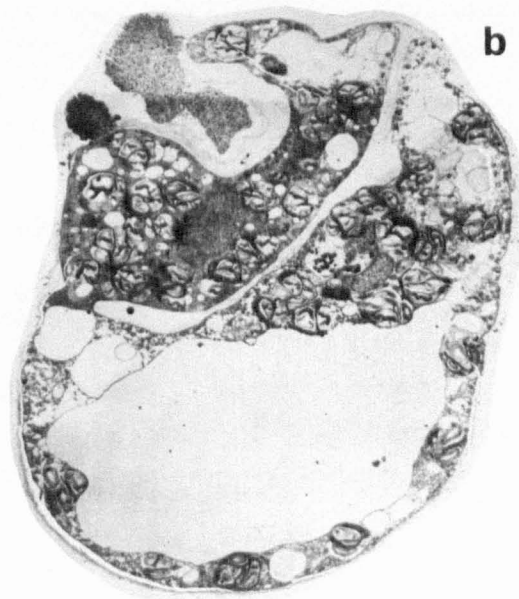
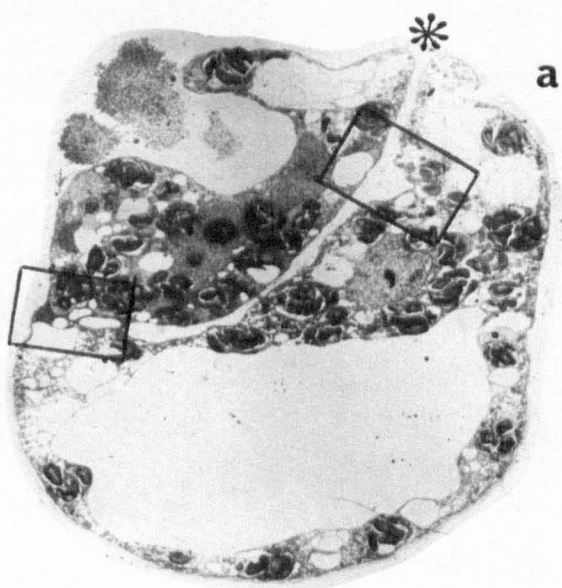
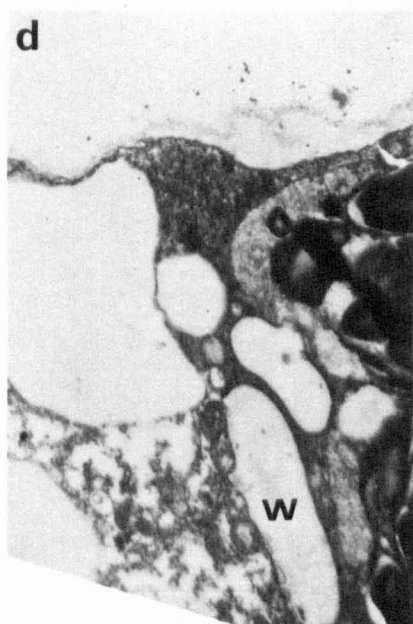
x 8,000

Figure 3.185 e A possible second wall (arrowed) forming from a
point on the cross wall, in Figure 3.185a.

x 7,500

Figure 3.185 f A detail of the cross wall outer wall junction in
Figure 3.185 c.

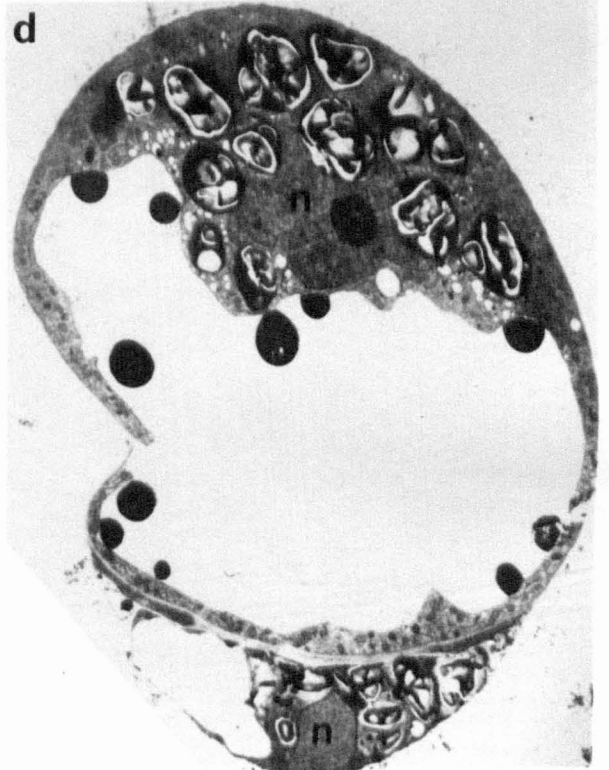
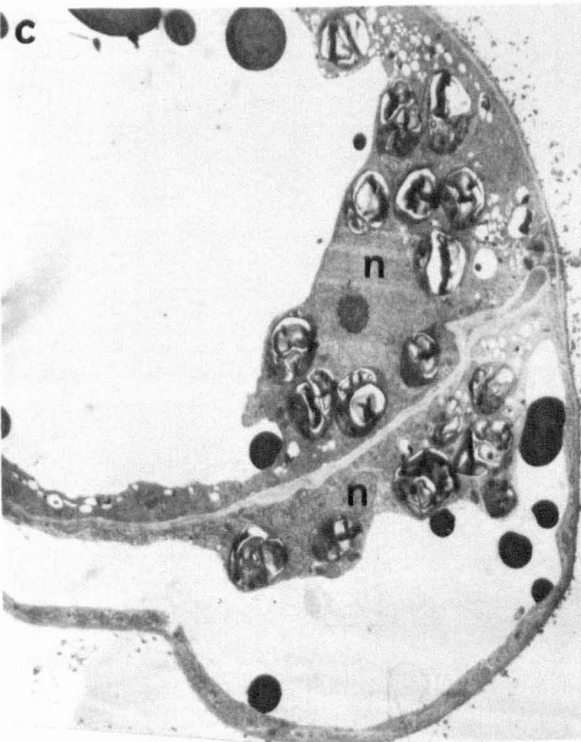
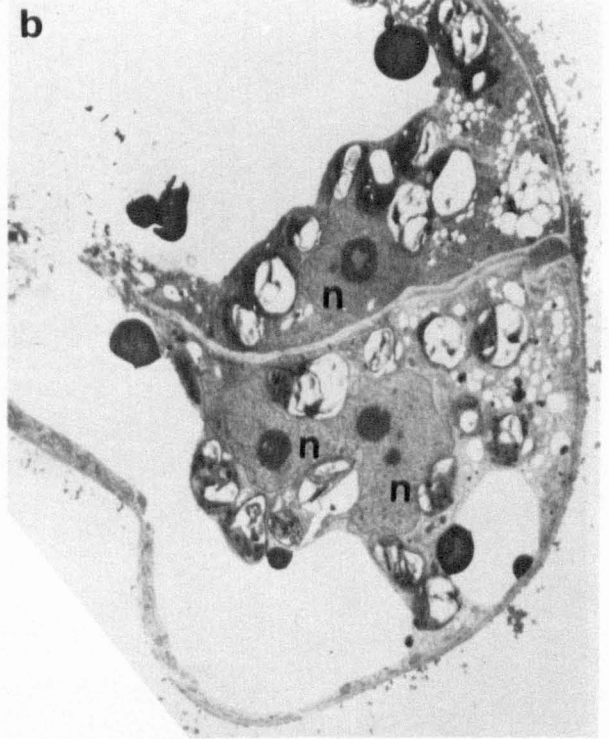
x 2,500



Division in a cultured spontaneous fusion body, (Cultured for 13 days.)

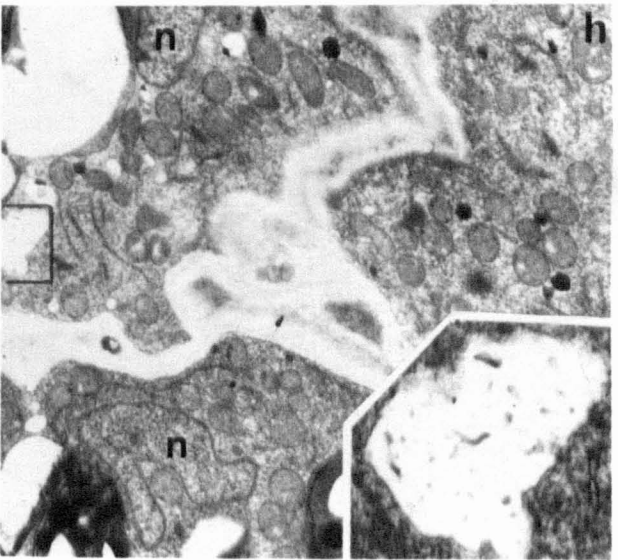
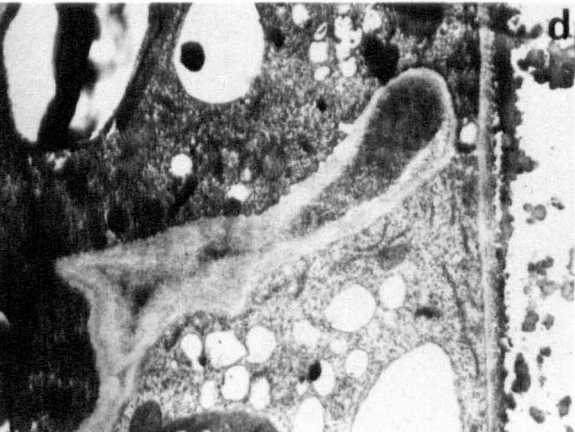
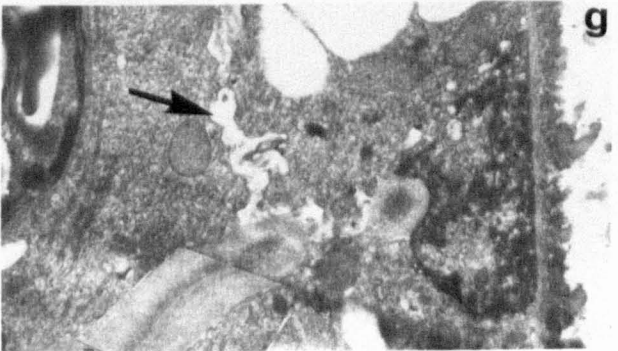
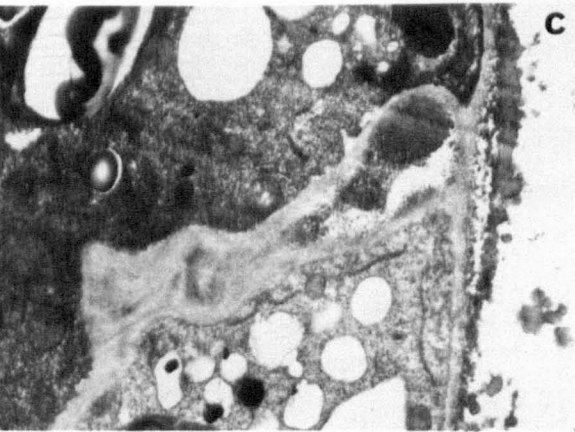
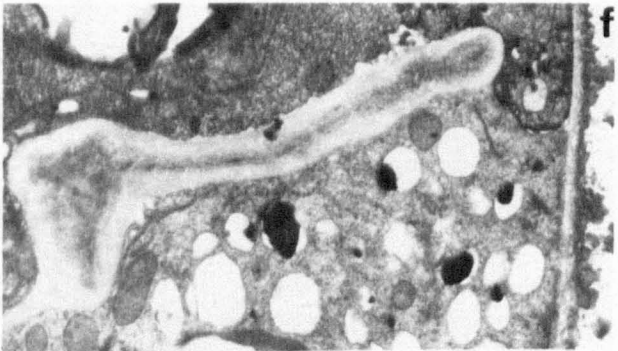
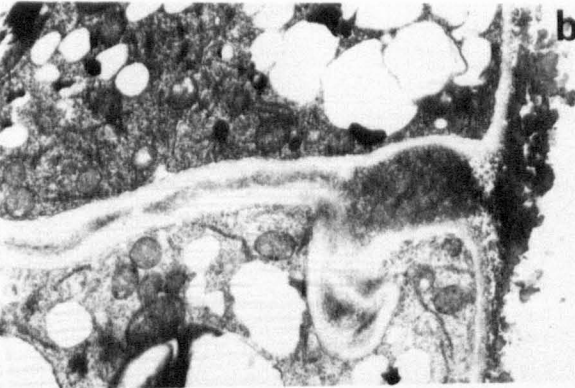
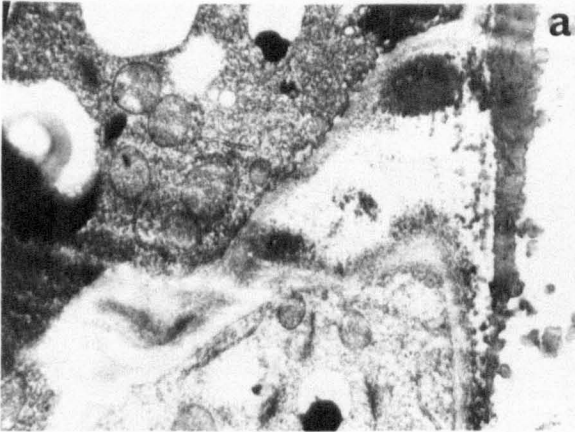
Figures 3.186 a to d Serial sections through the dividing fusion body. The dividing wall is initially tripartite, (Fig. 3.186 a) then becomes simple, passing obliquely downwards through the body. There are at least 5 nuclei present. Those within one area are closely associated. (Figures 3.186 a and b). Details of the junction of the cross wall with the outer wall are shown in the following figures. x 2,500

3.186



Division in a cultured spontaneous fusion body, details of the
cross wall outer wall junctions.

Figures 3.187 a to g Serial sections through the spontaneous fusion body shown in Figure 3.186. (e.g. sections 3.187 b and d correspond th Figures 3.186 b and c.) In the Figure 3.187 a the cross wall joins the outer wall with a broad junction. In Figures 3.187 b and c the connexion narrows. A dense,membrane bound structure lies between the cross and outer walls in Figures 3.187 d to g. In the latter figure an undulating (arrowed) wall appears to be running from the cross wall. Figure 3.187 h is a detail of the central complex of the cross walls in Figure 3.186a. A cosome (insert) lies near the wall junction. x 8,000

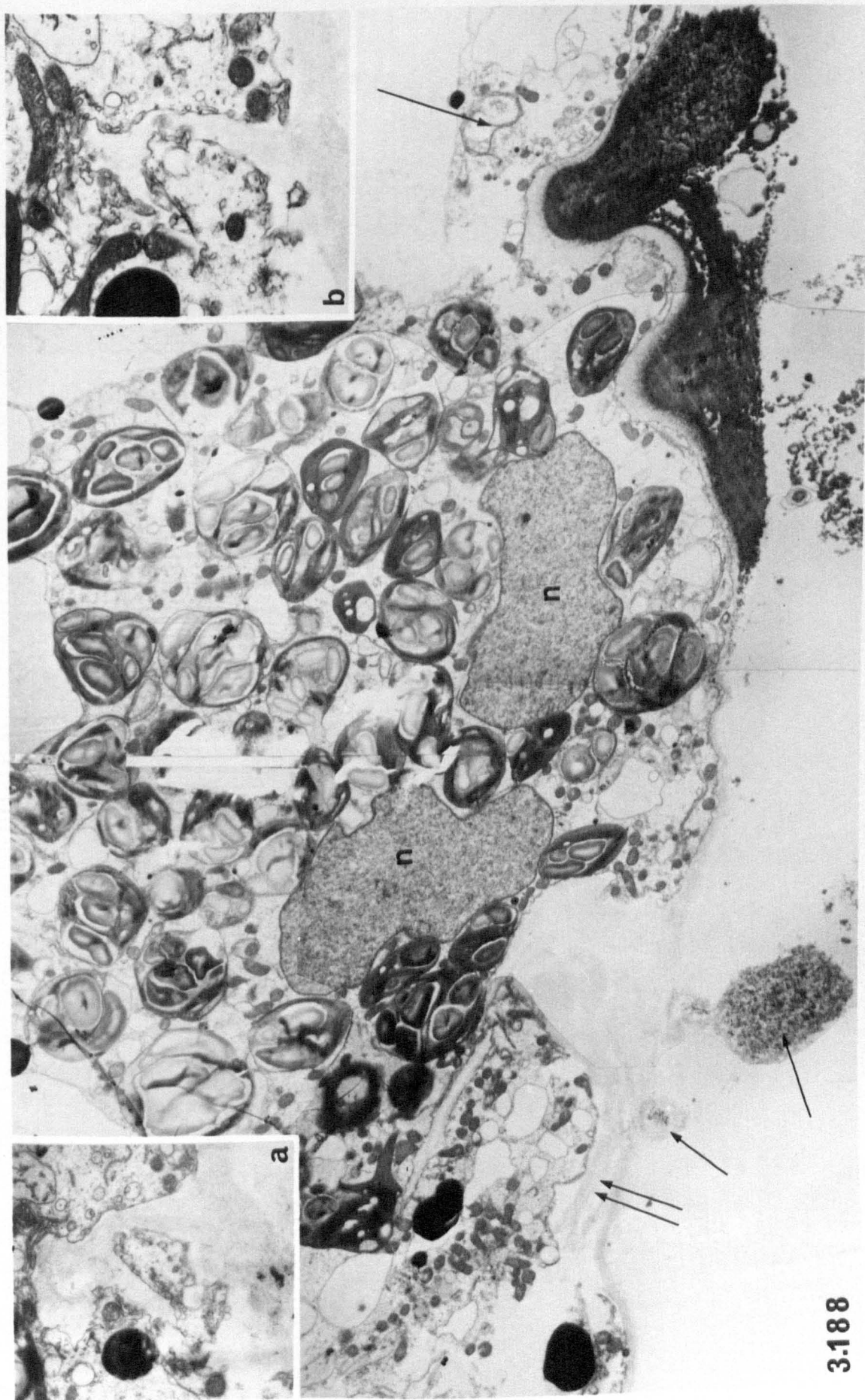


A cultured spontaneous fusion body .(Cultured for 13 days.)

Figure 3.188 A nucleated region of the spontaneous fusion body. To the left, two cosomes have been extruded. (arrows). A thickening in the wall extends as a narrow wall into the cytoplasm. The outer part of the wall is laminated (double arrows.) Chloroplast free, mitochondrial rich cytoplasm lies between the ingrowing and outer walls. The insets detail sections of the ingrowing wall from either side of the main section. To the right, two darkly stained cosomes lie in indentations in the body. Above this region, there is a membrane figure which suggests chloroplast breakdown. (arrow).

x 4,500

insets x 7,500



Summary

(Protoplast and fusion body culture)

The regeneration of colonies from uninucleate protoplasts, cultured on solid media, is described. Spontaneous fusion bodies cultured similarly failed to undergo any division, although some cytoplasmic reorganization and outer wall formation occurred.

In liquid media, however, spontaneous fusion body culture was more successful. Preparations containing both uninucleate protoplasts and fusion bodies were studied over a period of some three weeks, observations being made in the light and electron microscope.

In aspects of outer wall formation, the formation of vacuolar inclusions and some aspects of organelle reorganization, fusion bodies behaved similarly to uninucleates. In other respects they differed:

The fusion bodies showed an exaggerated tendency to form buds in the early stages of culture. These and peripheral regions of cytoplasm frequently became partitioned and subdivided. In general the pattern of internal wall formation was irregular. No definite mitotic divisions were observed, although nucleate as well as enucleate subunits were formed.

The nuclear associations which formed shortly after fusion body isolation involved the close juxtaposition of, usually, pairs of nuclei, which were connected by outer nuclear membrane bridges. However most of the associations dispersed in culture.

From the first stages of culture, fusion bodies contained cytoplasmic bodies termed cosomes. During culture these either formed an outer layer of wall like material or were expelled from the cytoplasm. The latter appeared to be necessary for long term fusion body viability. A general decline in the proportion of multinucleates was observed over the culture period.

3.5 QUANTITATIVE ASPECTS OF SPONTANEOUS FUSION.

All quantitative work was carried out using the tobacco mesophyll system. Results are either represented graphically or as condensed values derived from more extensive data. In both cases the original data are given in Appendix 3, the relevant data set being noted in the text.

3.5.1 Sample size determination. (Data Set 1)

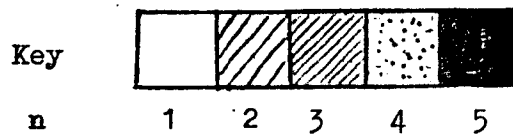
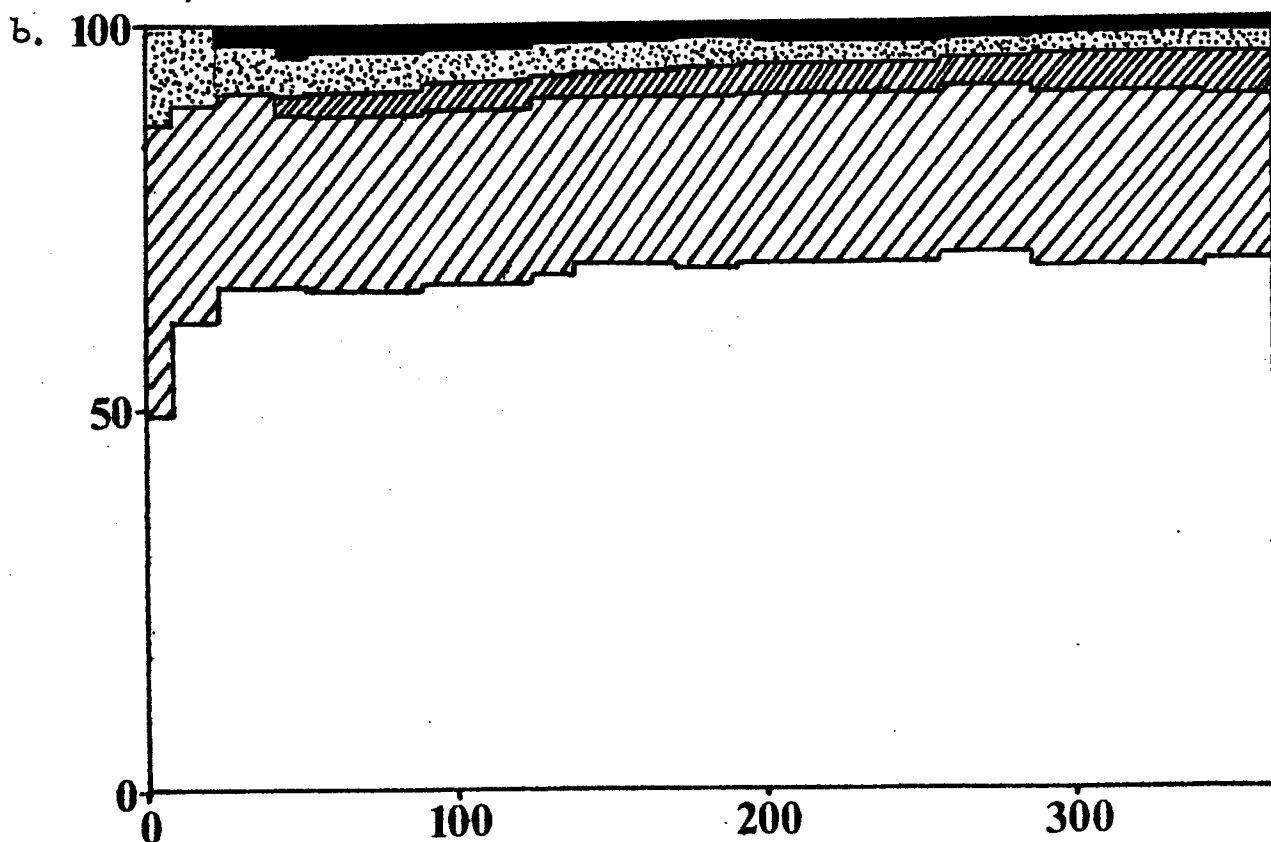
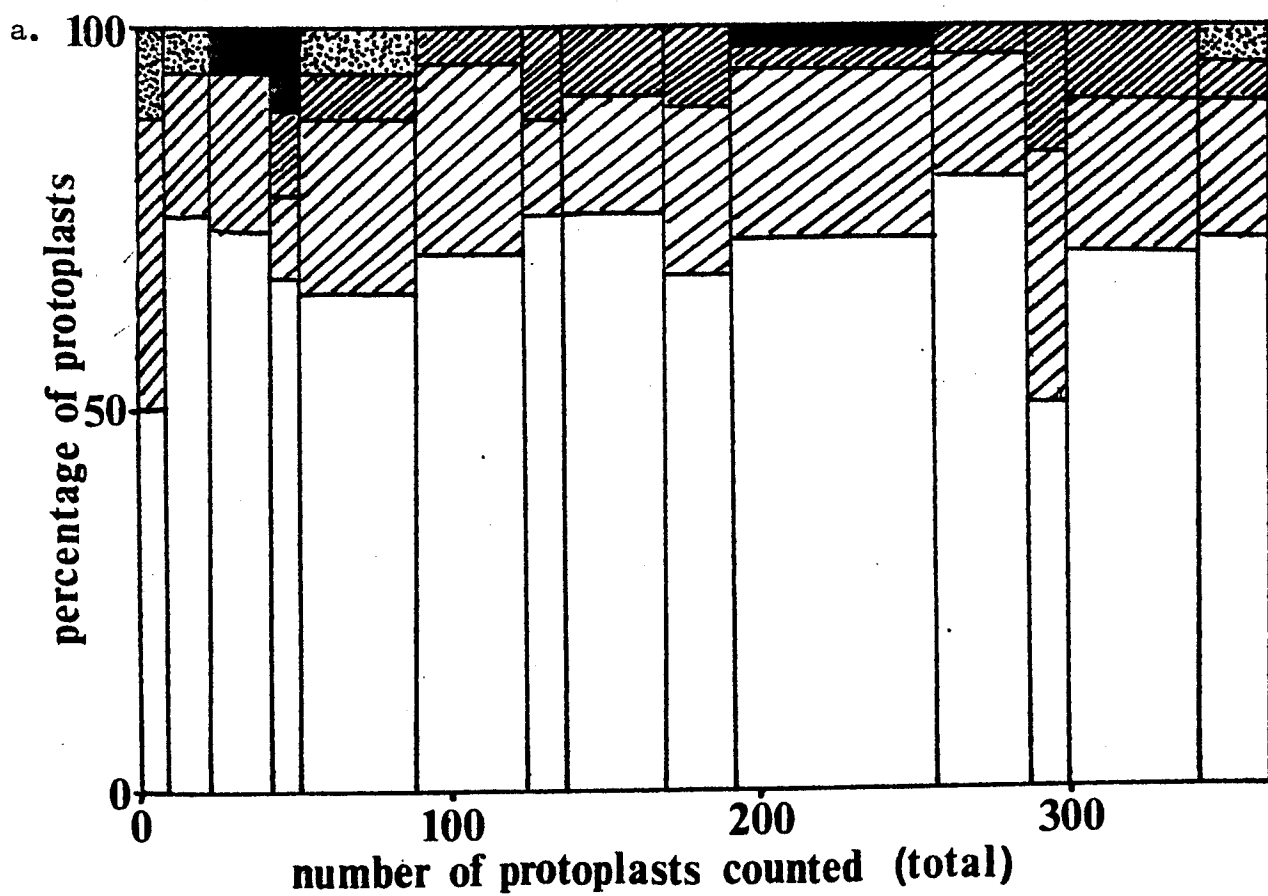
The minimum representative sample giving an accurate assessment of spontaneous fusion level, was determined as follows: The incidence of spontaneous fusion in 14 fields of a nuclear stained preparation was noted. Figure 3.189a expresses the results as percentages, with running means given in Figure 3.189b. The values rapidly stabilized. Sixty was considered an absolute minimum sample size, and where possible higher counts were made.

3.5.2 Methods of assessment of spontaneous fusion: a comparison.

Whilst considered definitive, nuclear counting of stained material was somewhat time consuming. Of the two methods, Feulgen staining provided the clearer, more easily counted, and more permanent preparation.

Direct observation of fresh material was rapid, but gave consistently low estimates of the level of spontaneous fusion when compared with nuclear stained preparations. For example, two preparations with 20.5% and 38.0% of multinucleates were estimated by direct observation to have only 15.0% and 32.5% respectively. (Data Set 2.)

3.189b Running mean percentages (axes as in 3.189a)



The measurement of photographs would appear to be rather less subjective. Three preparations were photographed upon isolation and then Feulgen stained. Mean protoplast diameters (arbitrary units) are compared with the corresponding percentages of multinucleates. (Table 3.1). (Data Set 3.)

Table 3.1

	Mean diameter	% age of multinucleates
1	6.3 ± 2.2	30.6
2	7.2 ± 4.0	40.0
3	4.9 ± 1.2	18.5

Although there is a good agreement between values a 33% difference in spontaneous fusion level (between 1 and 2) is represented by only a 14% difference in mean diameter with a low statistical probability of the samples being significantly different. (See Data Set 3.) In practice the method is both lengthy and tedious.

Feulgen staining was therefore adopted for routine assays.

An extension of nuclear counting uses microdensitometry to give values of nuclear DNA content. An advanced fusion bodies, where nuclear association makes direct counting difficult, microdensitometry has an obvious advantage.

The linearity of the DNA assay was tested using a Feulgen stained preparation. The nuclei were clearly defined, but grouped sufficiently closely to be assayed together. The DNA contents of single nuclei, and groups of 2, 3 and 4 were measured. The results are presented in Figure 3.190. (Data Set 4). There is a clear increase in the mean values (Fig. 3.191) with increase in the number of nuclei, but the 4 fold increase in

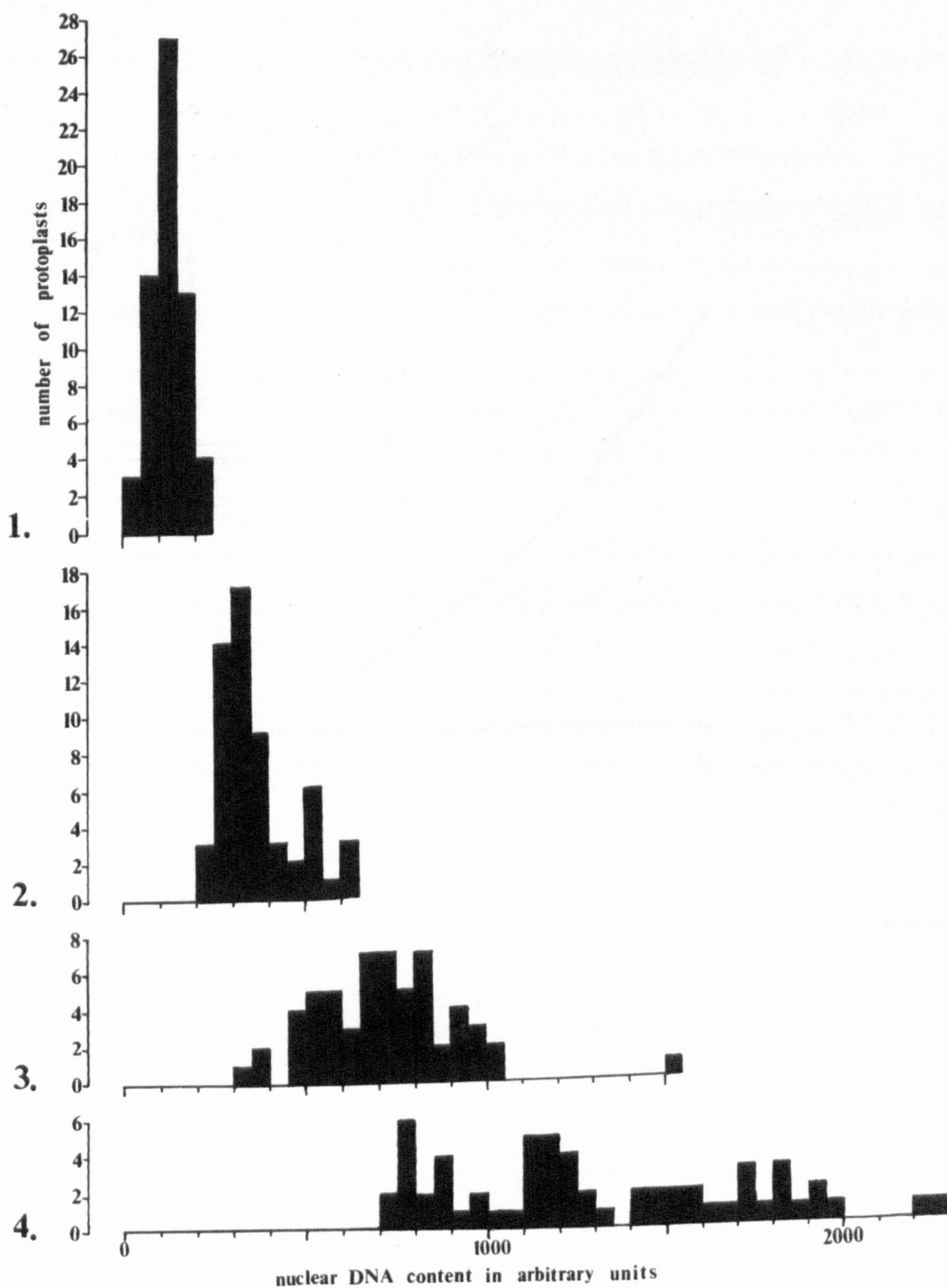


Figure 3.190 The DNA contents of populations of uni- bi- tri- and tetranucleate protoplasts. (Histograms 1, 2, 3 and 4, respectively.)

the latter gave a 10 fold increase in DNA level. Despite the overlap in the distributions of values all 4 sets are significantly different at a high level of probability. (Data Set 4.5).

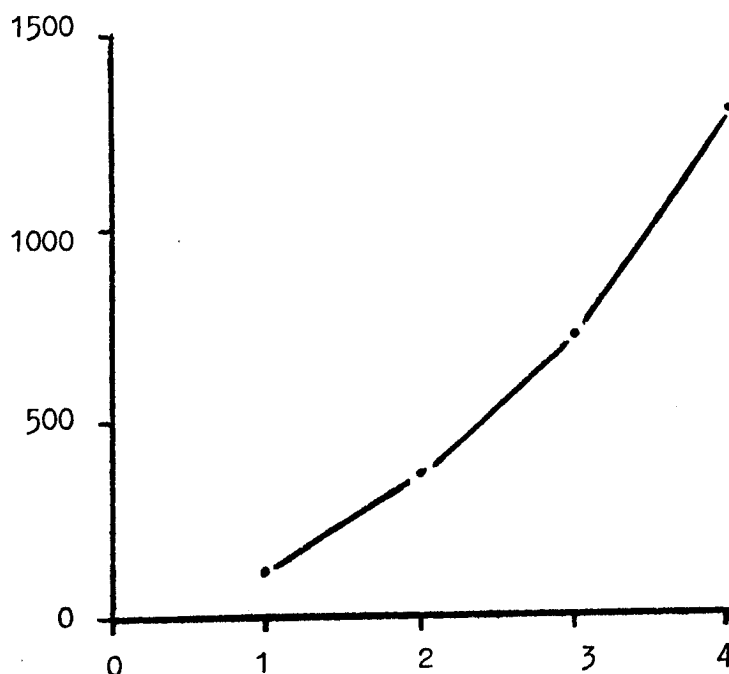


Fig. 3.191 Mean values of total nuclear DNA content (arbitrary units) plotted against number of nuclei.

Note: To determine the reliability of the microdensitometric assay, various controls were carried out. The reproducibility of one reading was tested by making 30 consecutive readings. (Data Set 5.1). The standard deviation of some 3% was acceptable. The calibration mechanism enabling correction for background density did not in fact give a satisfactory compensation. (Data Set 5.2). Therefore extreme compensation was avoided by a careful choice of backgrounds. Increasing mask size gave an increased measurement of DNA content, (Data Set 5.3) therefore one mask only was used throughout. Due to the inconsistency of Feulgen staining, comparisons were only made between preparations from the same staining batch.

3.5.3 An investigation of factors affecting the level of spontaneous fusion.

a. Osmotic conditions preceding protoplast isolation.

Routinely, stripped leaf pieces were placed directly in the enzyme incubation medium. By placing the pieces in osmoticum alone before the enzyme incubation medium the level of spontaneous fusion could be greatly reduced. Placing either the leaf pieces, or the whole leaf in water, had a similar effect. (Table 3.2, Data Set 6.)

Table 3.2

Pretreatment	% age of multinucleates
Control	20.6
Stripped leaf pieces placed in 13% sorbitol for 2h	12.0
Stripped leaf pieces placed in distilled water for 2h	6.6
Whole leaf placed in distilled water for 2h	7.3

b. Conditions during isolation: Enzyme and osmoticum concentration, temperature, additives.

A series of experiments was carried out using a range of enzyme concentrations (0.1 to 0.8% w/v Macerozyme and 1 to 8% w/v Meicelase P) and a range of osmoticum concentrations (10 to 16% w/v sorbitol). The specific conditions are given in Table 3.3. No accurate yields were calculated but relative yields are indicated.

Table 3.3

Preparation number	Osmoticum, % w/v sorbitol	Macerozyme % w/v	Meicelase P % w/v	Yield
1	13	0.1	4	++
2	13	0.2	4	++
3	13	0.6	4	++
4	13	0.1	1	+
5	13	0.4	8	+
6	13	0.8	4	++
7	13	0.4	4	+
8	10	0.4	4	++
9	16	0.4	4	++
10	16	0.8	4	+++
11	10	0.8	4	++
12	16	0.4	2	+++
13	10	0.4	2	+++

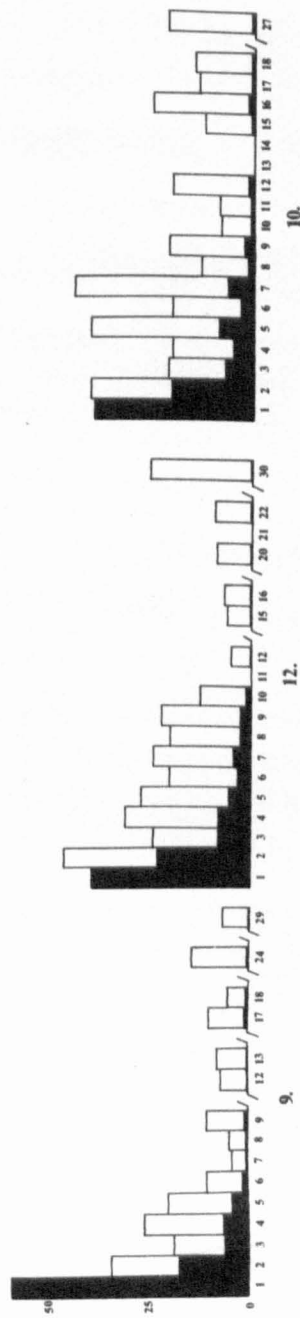
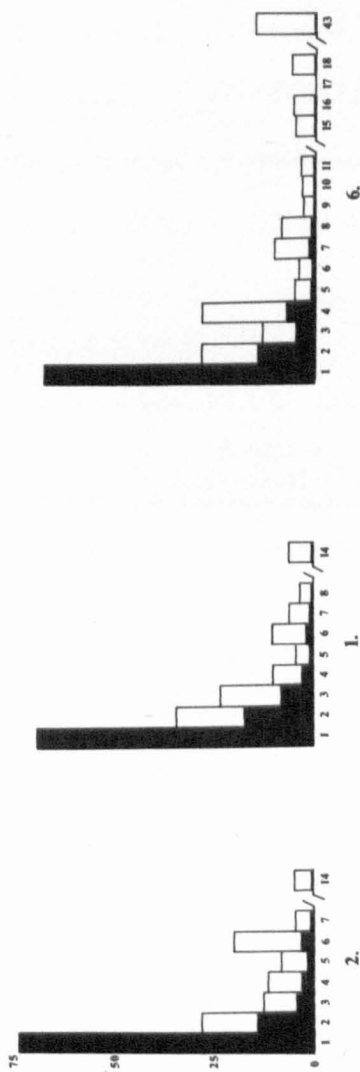
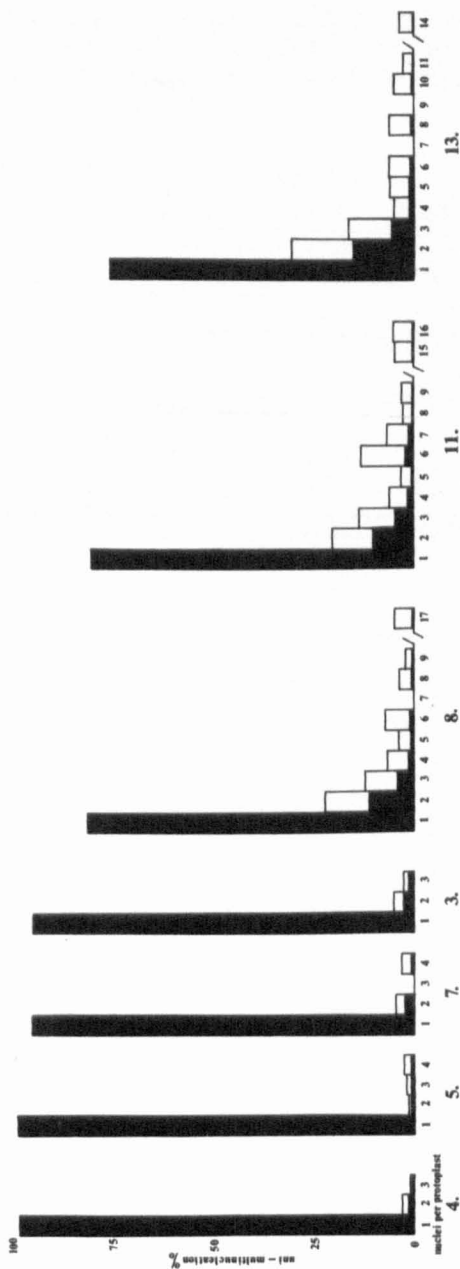
The preparations were Feulgen stained and nuclear counts carried out. The detailed results are given in Data Set 7, with calculations of the percentage of uninucleates, the percentage of the total number of nuclei in uninucleates and the mean number of nuclei per protoplast. Histograms derived from the results are shown in Figure 3.192, in order of increasing multinucleation. The solid blocks represent the percentage of protoplasts with each number of nuclei. The open blocks indicate relative numbers of nuclei in each of the groups of multinucleates thereby giving an indication of the distribution of nuclei in each system. No generalised conclusions can be made; the results are considered further in Section 6.3.1.

There is a clear relationship between the mean number of nuclei per protoplast and the level of uninucleation expressed either as the percentage of uninucleates or the percentage of nuclei in uninucleates.

O

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Figure 3.192 Histograms showing the %age yield of uninucleate and multinucleate protoplasts from tobacco mesophyll tissue treated with various enzyme incubation media. (See table 3.3) Open blocks indicate the number of nuclei in the multinucleates. The latter values do not relate to the %age scales indicated to the left of the figure. The scale differs for each histogram.



(numbers beneath histograms refer to preparation numbers given in Table 3.3)

(Fig. 3.193). The significance of this is, that the most easily calculated value, the percentage of uninucleates, would appear to define the preparation in question. Thus the data collection can be greatly simplified. Additional points on the graph are taken from an assay of a preparation produced from 50 day old plant material (Δ and \times) and from data relating to multinucleation in White Burley variety tobacco leaf protoplasts (\bullet and \circ) (Evans, Woodcock and Keats, 1973).

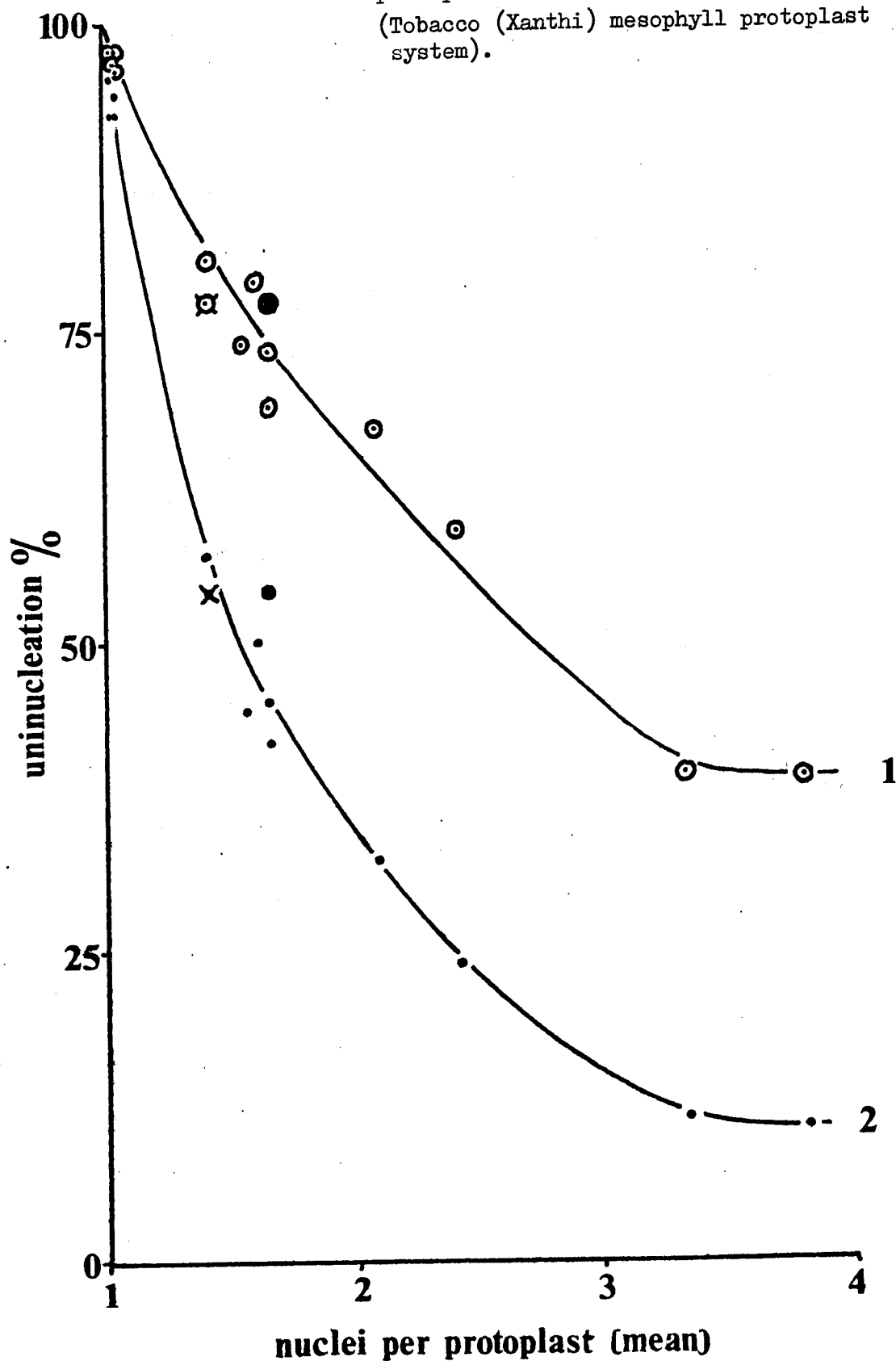
Purified Meicelase P was compared with equivalent amounts of crude enzyme. (Taking the increase in activity by purification to be approximately 10 fold : P. K. Evans, personal communication). Table 3.4 shows the respective yields of multinucleates. (Data Set 8).

Table 3.4

Enzyme incubation medium (plus 13% w/v sorbitol)			% age of multinucleates
Meicelase P % w/v		Macerozyme % w/v	
Crude	Purified		
2		0.4	
	0.2	0.4	
4		0.4	
	0.4	0.4	
10		0.4	
	1.0	0.4	

The purified enzyme preparation produced a more rapid leaf digestion in all cases, with some increase in multinucleation. Higher levels of purified enzyme were deleterious.

Figure 3.193 Graphs showing 1. % age of uninucleate protoplasts plotted against mean number of nuclei per protoplast.
 2. % age of nuclei in uninucleate protoplasts plotted against mean number of nuclei per protoplast
 (Tobacco (Xanthi) mesophyll protoplast system).



As mentioned in Materials and Methods (2.2.1), the enzymes are routinely used below their temperature optima. Preparations containing 0.4% Macerozyme, 4% Meicelase P and 13% sorbitol, were incubated overnight at 20°C, 30°C and 40°C. Leaf material from 50 and 82 day old plants was used. In all cases, increase in temperature produced a more rapid tissue digestion, but only in the older tissue was there any significant increase in multinucleation. The results are presented below in Table 3.5. (Data Set 9).

Table 3.5

Age of plant material, days	Temperature °C	% age of multinucleates
50	20	8.9
50	30	10.3
50	40	9.7
82	20	15.8
82	30	25.0
82	40	36.1 *

The protoplasts produced at 40°C were all highly unstable. The spontaneous fusion bodies produced from the 82 day old material* were mostly flat and failed to round off with time.

Culture medium (TMS) was added, instead of water in some preparations, to make up the volume of the enzyme incubation medium. Very little change in the level of spontaneous fusion was produced, but the bodies were more stable, rounded off better and the overall yield of protoplasts was increased in the presence of TMS. The results from two comparisons are given in Table 3.6. (Data Set 10).

Table 3.6

Enzyme incubation medium		% age of multinucleates		Yield, X 10 ⁶ protoplasts/g
0.4% w/v Macerozyme 4% w/v Meicelase P	distilled water	1.	24.3	0.99
		2.	18.7	0.85
13% w/v sorbitol	TMS	1.	26.1	1.30
		2.	19.4	1.28

Sodium nitrate (1.75 % w/v) was added to the enzyme incubation medium, partially substituting for sorbitol as the osmoticum. The effect was extremely deleterious, the yield of live protoplasts being reduced too much for any analysis to be carried out.

Cytochalasin B at levels of 5 μ g/ml to 100 μ g/ml was added to the enzyme incubation medium. Over 20 μ g/ml was highly deleterious. At a level of 10 μ g/ml there was initially a marked increase in the number of large flat fusion bodies, but in all preparations a large proportion of the yield failed to survive washing procedures and therefore no analysis of the yields was carried out.

- c. Age of plant material (The above experiments used plants of approximately 80 days, except where stated).

Leaf material from tobacco plants aged 44 to 89 days was treated with enzyme incubation medium (containing 0.4% Macerozyme, 4% Meicelase P, 13% sorbitol). The degree of tissue digestion and the yield of live material varied between preparations. Therefore absolute yields as well as percentages of uninucleates and multinucleates were counted. The results are presented in Figure 3.194. (Date Set 11). The yield of protoplasts peaks sharply at 75 days,

Changes in the level of spontaneous fusion body production with age of plant material. (Tobacco (Xanthi)). (Yields are expressed as percentages of 75 day values).

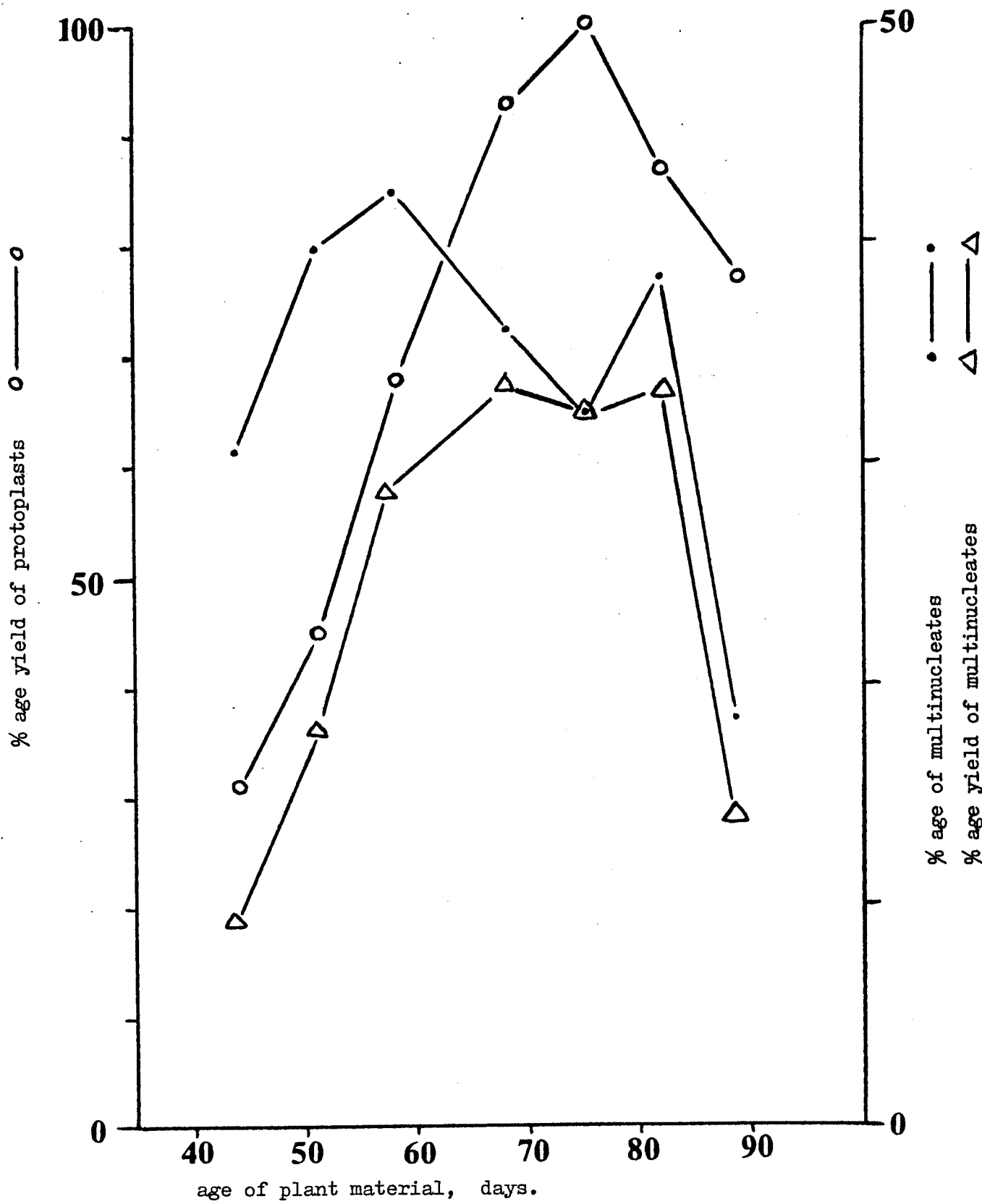


Figure 3.194 . — . % age of multinucleates plotted against age of plant matl.
o — o % age yield of protoplasts " " " " " "
Δ — Δ % age yield of multinucleates " " " " " "

whilst, the maximum number of multinucleates is produced from 68 to 82 days.

3.5.4 Changes in the level of multinucleation during culture.

The level of multinucleation was measured by nuclear counting at three stages in the culture of two spontaneous fusion body containing preparations. The results are given in Table 3.7. (Data Set 12).

Table 3.7

	% age of multinucleates	
	Culture 1	Culture 2
Day 0 (freshly isolated)	22.5	21.8
Day 12	7.5	
15		9.6
20	3.4	
23		2.8

As evidenced in the electron microscopic study of cultured fusion bodies most nuclei are sufficiently dissociated to allow individual counting. However those which appear to be undergoing division are still closely associated after some time in culture. A microdensitometric study was carried out to determine the range of DNA contents in cultured preparations.

A control, uninucleate culture was assayed at 14 days. The distribution of DNA values is shown in Figure 3.195, histogram 1, (Data

Set 13). The mean DNA value is 457. Two fusion body cultures (as used above, Table 3.7) were assayed at 12 and 15 days. They produced mean DNA values of 652 and 750 respectively.

In an attempt to pick out cells possibly derived from spontaneous fusion bodies, half of the readings from each culture were of nuclei in single or paired cells and half the readings were of the nuclei in clumped cells. (As mentioned in Section 3.4.2 spontaneous fusion bodies tend to form pseudo-colonies at an early stage of culture). The two samples of "single" cells give mean DNA values of 289 and 343 whilst the "clumped" cells gave values of 1014 and 1146. The relevant distribution of DNA values are shown in Figure 3.195, histograms, 2, 4, 3 and 5 respectively. (Data Set 14).

Because of the manner of selection of the samples, no statistical analysis was applied.

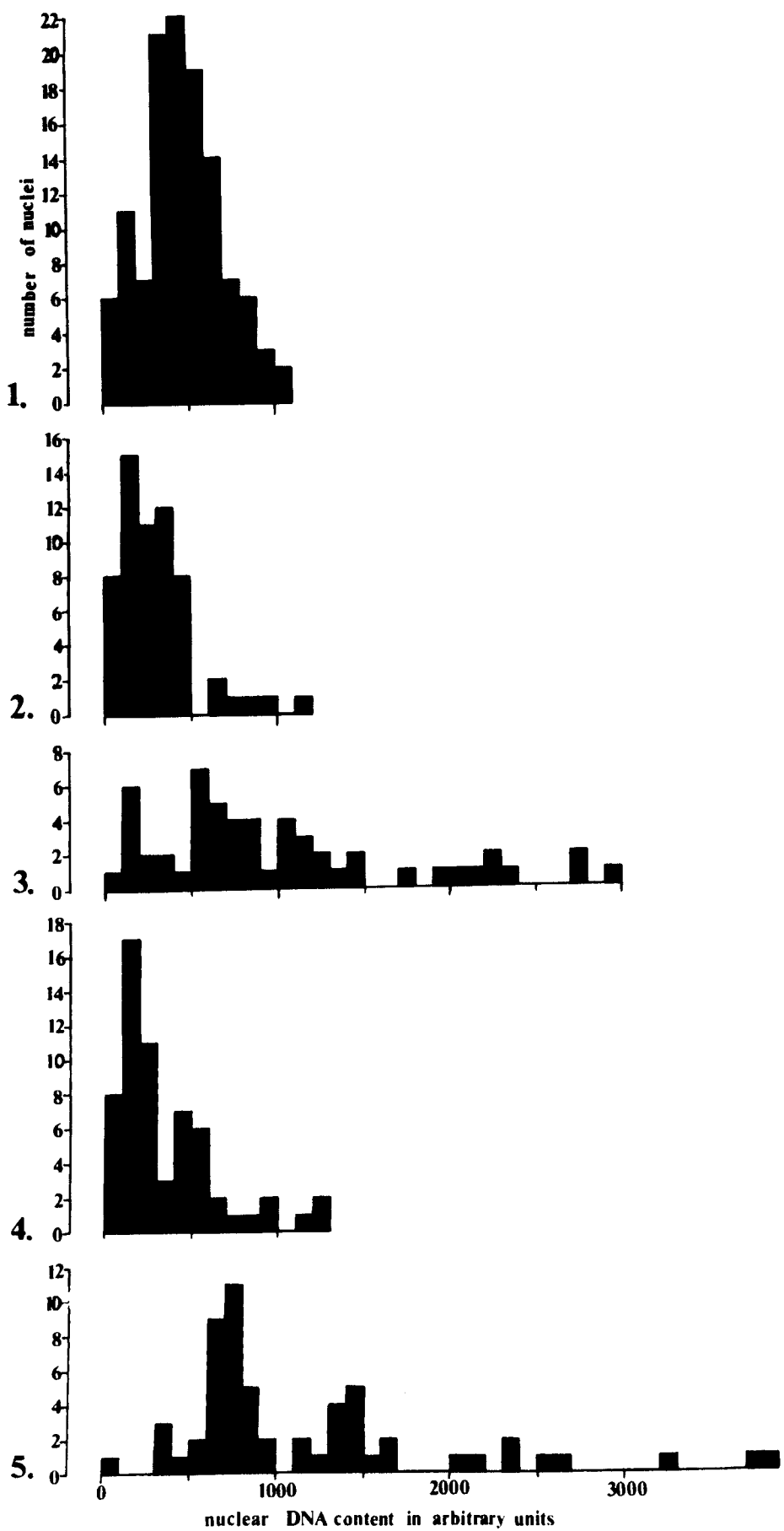
Figure 3.195, Histogram 1: The range of DNA contents in a control culture of tobacco mesophyll protoplasts.(14 days in culture.)

2: The range of DNA contents in a 12 day culture of a spontaneous fusion body containing preparation.
a. 'single' cells.

3: The range of DNA contents in a 15 day culture of a spontaneous fusion body containing preparation.
a. 'single' cells.

4: As Histogram 2 ,but:
b. 'clumped' cells.

5: As Histogram 3 ,but:
b. 'clumped' cells.



Summary

(Quantitative aspects)

The several methods for the assessment of the level of spontaneous fusion, described in section 2.4, were compared. Feulgen staining alone was considered to give a reliable estimate.

A microdensitometric assay of DNA contents in uni-, bi-, tri- and tetranucleate protoplasts demonstrated that the method could be used to identify large populations of multinucleates, useful, for example, when the degree of nuclear aggregation makes optical counting difficult or impossible.

The effect of conditions preceding and during protoplast isolation on the yield of multinucleates was investigated. The osmotic conditions prior to isolation clearly affect the level of fusion, as do the osmotic and other conditions during isolation. However none of the effects is easily explained. In older tissues, the temperature at which the isolation was carried out could affect the level of spontaneous fusion. Increase in fusion level with temperature was accompanied by an increase in the rate of tissue digestion. Increase in the latter by the use of purified enzymes does not, however, increase the level of fusion.

The inclusion of TMS medium in the enzyme incubation medium increased the overall yields of spontaneous fusion bodies but only by increasing the yield of all protoplasts. I.E. the proportion of fusion bodies was not increased. The age of plant material used affects both the proportional and overall yields of spontaneous fusion bodies.

A relationship between the level of multinucleation and the mean number of nuclei per protoplast, enabled a reduction in the amount of data collection necessary to accurately determine the level of fusion in a preparation.

The level of multinucleation in two spontaneous fusion body containing preparations was assessed upon isolation and during culture. Over a three week period the level dropped considerably. DNA assays of the two cultures were compared with a similarly aged uninucleate preparation. The values obtained by the former differed greatly from the latter in terms of pattern of distribution of values and absolute values.

CHAPTER FOUR

THE INDUCED ADHESION AND FUSION OF PROTOPLASTS:

MATERIALS AND METHODS

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(Suppliers of non-standard chemicals are listed in text)

4.1 GROWTH OF PLANT MATERIAL

4.1.1 Tobacco, i.

Plants of Nicotiana tabacum cv Xanthi NC were grown as described in Section 2.1.4. Leaf material was harvested at 75 to 85 days. (See 2.2.5).

4.1.2 Tobacco, ii.

Plants of Nicotiana tabacum cv Bright Yellows were grown as described in Section 2.1.4. They were infected with Tobacco Mosaic Virus (TMV) to induce leaf chlorosis. Leaf material was selected by growth stage.

4.1.3 Petunia

Plants of Petunia hybrida cv Blue Dandy were grown under essentially the same conditions as described in Section 2.1.4, but leaf material was harvested at 40 to 50 days, omitting the final transplant.

4.2 PROTOPLAST ISOLATION

4.2.1 Enzymes

The enzymes described in Section 2.2.1 were used in the preparation of enzyme incubation media.

4.2.2 Tobacco leaf protoplast isolation, i.

Protoplasts were isolated from both the mesophyll and epidermes of the Xanthi leaf. The lower epidermis was removed, preplasmolyzed (see 4.3.1) and then placed in the enzyme incubation medium. The stripped leaf pieces were preplasmolyzed then incubated separately. Digestion

progressed to completion and the protoplasts were washed several times in sorbitol to separate the mesophyll and epidermal protoplasts. Three flotations in sorbitol were sufficient to give a virtually 100% separation. The upper and lower epidermis protoplasts were bulked.

Enzyme incubation medium

0.4%	w/v	Macerozyme
4%	w/v	Meicelase P
13%	w/v	sorbitol

4.2.3 Tobacco leaf protoplast isolation, ii.

Chlorosed regions of the Bright Yellows leaf were stripped, preplasmolyzed and placed in the enzyme incubation medium. The protoplasts were washed several times, it being necessary to centrifugally concentrate them at each washing due to the very low yields.

Enzyme incubation medium.

0.5%	w/v	Macerozyme
5%	w/v	Onozuka P3000
23%	w/v	sucrose

4.2.4 Petunia leaf protoplast isolation

Stripped pieces of petunia leaf were preplasmolyzed then placed in enzyme incubation medium. The mesophyll protoplasts released were washed several times.

Enzyme incubation medium

0.5%	w/v	Macerozyme
5%	w/v	Onozuka P1500
20%	w/v	sucrose.

4.3 CONTROL CONDITIONS

Although not necessarily ideal in some respects, the non-dividing leaf tissue was used to prevent ambiguities due to mitosis. To satisfactorily exclude any spontaneous fusion effects, two controls were incorporated in the experiments:-

4.3.1 Preplasmolysis

By placing stripped leaf pieces in a solution of the osmoticum in use, it was possible to reduce the level of spontaneous fusion. (See 3.5.3). A 4h treatment effectively eliminated it. Preplasmolysis in culture medium plus osmoticum rather than osmoticum alone produced healthier protoplasts.

Although used routinely, preplasmolysis proved to be necessary only with the tobacco mesophyll system, spontaneous fusion being virtually absent in the other systems.

4.3.2 Markers

In the light microscope, Xanthi tobacco epidermal protoplasts are readily distinguished by size and colour from Xanthi mesophyll and petunia mesophyll protoplasts, as are the Bright Yellows protoplasts. In the electron microscope, fine structural differences can be used to identify all of the protoplasts. The sparse and poorly developed chloroplasts and occasionally the presence of TMV identify Bright Yellows protoplasts (Figs. 5.4a and 5.4b). The degree of vesiculation, the few, small systrophied chloroplasts and frequency of golgi bodies distinguish the epidermal protoplasts from the Xanthi and petunia mesophyll protoplasts. (See Appendix 2.2). Slight chloroplast differences are observed between

the latter two, but these are considered unreliable.

(See also Appendix 2.1)

4.4 INDUCING AGENTS

4.4.1 Inorganic salts.

The effects of a number of inorganic salts were studied in detail. The concentrations used were based on osmosity, to ensure that osmotic disturbance of the protoplasts was minimal. The molarities with an osmosity equivalent to that of 0.77 M sucrose (24% w/v) are given in Table 4.1. Where lower concentrations of salts were desired, sugars were used to compensate osmotically.

Table 4.1 The molarities of selected inorganic salts giving an osmosity equivalent to 0.77 M sucrose.

Salt	Molarity
sodium nitrate	0.61
sodium nitrite	0.60
sodium chloride	0.56
potassium nitrate	0.67
potassium chloride	0.55
calcium nitrate	0.42
calcium chloride	0.37
ammonium nitrate	0.62
ammonium chloride	0.57
ammonium sulphate	0.55

Details of salt treatment are given in Section 5.1.1. Less intensive studies were carried out using several other salts. These are listed in Section 5.1 . 2., Table 5.1.

4.4.2 Organic compounds, i.

Protoplasts were treated with aqueous solutions of the following

organic compounds:

Urea
 lysozyme (Sigma Chemical Co.)
 Concanavalin A (Con A) (Miles Yeda)
 1AA (Sigma Chemical Co.)
 24D (Sigma Chemical Co.)

Where necessary an osmoticum was added to the solutions. Details of treatment are given in Section 5.2.

4.4.3 Organic compounds, ii.

Emulsions consisting of either a salt or sugar as the aqueous phase and one or more of the following compounds as the non aqueous phase were used to treat protoplasts:-

lysolecithin (Lipid Products)
 glyceryl monooleate (Monolein; Sigma Chemical Co.)
 glyceryl dioleate (KK Laboratories, California)
 glyceryl trioleate (Triolein; Sigma Chemical Co.)
 lecithin (Lipid Products)
 oleic acid (B.D.H.)
 retinol (Synthetic Vitamin A Alcohol; Roche Products)

The emulsions were prepared by one of three methods:

- a. manual stirring or shaking.
- b. using a vortex mixer.
- c. Using an ultrasonic disintegrator (M.S.E. ultrasonicator).

Appropriate mixtures were placed in a conical flask. A stream of nitrogen was directed from a gas cylinder on to the surface of the liquid. The height of the glass was adjusted to locate the probe (8 mm diameter) just below the surface of the liquid. The ultrasonicator was switched on and tuned to give the maximum intensity. Two to four minutes of ultrasonication was found to be sufficient. Smaller vessels

were cooled during the preparation of the emulsions.

Emulsions were used within 20 min of preparation. Details of the combinations in which the compounds were used and details of protoplast treatment are given in Section 5.3.

(Lysolecithin was also used in aqueous solutions.)

4.4.4 Sendai Virus

Three isolates of Sendai Virus were used to treat protoplasts:-

- a. Commercially prepared virus (Microbiological Associates; Bethesda, Maryland).
- b. Laboratory cultured virus, (Donated by Dr. G. B. Clements)
- c. Laboratory cultured virus (Prepared at Nottingham - seed virus donated by Dr. G. B. Clements).

The latter isolate was prepared as follows: Ten day old embryonated eggs were inoculated with one drop of seed virus of titre 40,000 HAU. After 3 days incubation at 37°C, the eggs were cooled to 4°C to kill the embryos and minimize bleeding. The egg shell and underlying membranes removed, the allantoic fluid was pipetted off. Debris was removed by centrifugation at 3,000 rpm for 5 min. The supernatant was then centrifuged for 60 min at 20,000 rpm, having reserved a little for the haemagglutination titration.

The titration was carried out twice, once using a wide range of dilutions and the second time using a narrow range to more accurately determine the virus concentration. 1ml of allantoic fluid diluted with Phosphate Buffered Saline (PBS) was added to 1 ml of a 1% suspension of Guinea Pig Red Blood cells in PBS. Controls using "neat" allantoic fluid

and PBS alone were also prepared. The mixtures were incubated for 2 h at room temperature and then overnight at 4°C. The first titration gave a concentration of between 2,560 and 5,120 HAU/ml and the second, a concentration of 5,000 HAU/ml.

The centrifuged pellet of virus was resuspended in sufficient PBS (plus 5% w/v Bovine Serum Albumin (Pentex Ltd.)) to give a concentration of 20,000 HAU/ml. The virus was stored, in 2 ml sealed ampules, under liquid nitrogen at -197°C.

Before use, some of the virus was inactivated using β -propiolactone (Sigma Chemical Co.) (Neff & Enders, 1968). A 10% w/v solution of β -propiolactone (BPL) was diluted by 1/20 with a solution of 1.68 g of sodium bicarbonate and 0.5 ml of 0.4% w/v phenol red in 100 ml of 0.6M sodium chloride. The solution (now 0.5% w/v of BPL) was cooled, the virus suspension being used whilst still cool after thawing. One part of BPL was added to 9 parts of virus suspension and the mixture placed in a tightly stoppered flask, at 4°C for 10 min, shaking frequently. The virus was stored overnight at 4°C.

The virus was used within 48h of thawing. Its capacity to fuse animal cells was tested using Hamster (BHK) cells.

Details of the treatment of protoplasts with Sendai Virus are given in Section 5.4.

4.5 CULTURE OF PROTOPLASTS

4.5.1 Aseptic techniques

The precautions described in Sections 2.3.1 and 2.3.2 were

observed.

4.5.2 Culture media

With few exceptions, protoplasts were cultured in solid media based on Modified White's medium. (Table 2.2).

4.6 EXAMINATION OF MATERIAL

4.6.1 Light microscopy

The light microscopes and techniques described in Section 2.5 were used.

4.6.2 Electron microscopy

Material was prepared for electron microscopy as described in Sections 2.6.2 to 2.6.5. Thin sections were viewed in the "EM6B" and "Corinth" electron microscopes. Replicas of freeze-etched material (see below) were also examined.

4.6.3 Freeze-etching

Drops of material, suspended in a suitable osmoticum, were placed on collared, gold specimen holders and frozen by immersion in Freon 22 at its freezing point. The freeze etching was carried out in a Balzers 360M Freeze Etcher using a technique based on that of Moor and Mühlethaler (1963). The specimens were fractured at -100°C with an etching time of 1 min. Platinum/carbon was evaporated on to the specimens from a resistance heated carbon arc for 15 sec. The replicas were floated from the holders on to water and cleaned using commercial bleach, 70% sulphuric acid and several changes of distilled water. They

were then mounted on formvar coated grids.

No fixatives or cryoprotectants were used, so as to eliminate preparative artifacts (Grout, Willison and Cocking, 1972).

Summary

(Chapter Four)

Techniques for the isolation of Tobacco (Xanthi), Tobacco (Bright Yellows) and Petunia protoplasts are described. Control techniques, involving the use of pre-plasmolysis to reduce spontaneous fusion and the use of light and electron microscopic markers to differentiate between spontaneous and induced fusion are described.

A number of adhesion and fusion inducing agents were employed, including inorganic salts (Table 4.1) certain organic compounds, including lysozyme and Concanavalin A, lipid emulsions and Sendai virus. Details of the preparation of the lipid emulsions and the culture of the virus are given.

Protoplast culture techniques and microscopical techniques have already been covered in detail (Chapter Two), with the exception of freeze-etching. The preparation of freeze-etch replicas is therefore described.

CHAPTER FIVE

THE INDUCED ADHESION AND FUSION OF PROTOPLASTS

RESULTS

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5.1 THE TREATMENT OF PROTOPLASTS WITH INORGANIC SALTS

5.1.1 General Protocol

Following isolation and washing, protoplasts were treated with salt solutions of varying concentrations. The salts were used: (a) in the molarities listed in Table 4.1: (b) in halved molarities, the solutions being made up to the desired osmoticity with a sugar: and (c) in the case of sodium nitrate, in a wide range of concentrations from 0.61M to 0.05M. Some salt combinations were also used, these are listed at the bottom of Table 5.1.

The protoplasts were allowed to settle in the washing medium, which was then replaced by the salt containing solution. The protoplasts sank, due to the low relative densities of the solutions. Centrifugation was occasionally used but was not essential. After the desired treatment duration, the salt solutions were replaced by a washing medium of either sorbitol to maintain protoplast compaction or sucrose to cause the protoplasts to float.

5.1.2 Individual salt effects, light microscopic observations.

Ideally a quantitative study would have been carried out, comparing various salt treatments. However, for various reasons this was not applicable. No direct measure of force of adhesion between protoplasts was possible and frequently the handling of protoplasts for light microscopy disturbed them sufficiently to invalidate observations. Therefore comparisons between different salt treatments were based on the response of the protoplasts to certain tests applied at specific stages in the salt treatments.

Typical effects induced by salt treatment are described and then a

"table of activity" evaluating the relative salt effects is constructed. (Table 5.1).

All of the salts listed in Table 4.1 induced protoplast adhesion virtually immediately. Observation of the protoplasts at this stage showed the formation of aggregates in which adjacent protoplasts appeared tightly adpressed. The adhesion was maintained in the presence of the salts. Replacement of the salt solutions by a sucrose solution rapidly induced flotation. The mechanical disturbance, presumably aided by surface tension effects, had a marked effect on the protoplast aggregates. Flotation after 5 min salt treatment dispersed many of the aggregates. The dispersed protoplasts were seen to float to the surface individually. After longer salt treatment the protoplasts floated to the surface more slowly in a cohesive strand many aggregates remaining intact. Prolonged treatment produced a sticky mass of protoplasts which were difficult to disperse without producing considerable damage.

After even brief treatments with some of the salts, protoplasts were very susceptible to mechanical damage, stripping off the plasmalemma and cytoplasm from the vacuole occurring. (Schenk, 1971). Many of the single protoplasts in such preparations were observed to have a "halo" of diffuse material, whilst within aggregates some of the protoplasts adhered closely as described above, others appearing linked but clearly not by direct membrane contact.

It was found to be possible to minimise damage whilst producing the maximum protoplast adhesion by treating protoplasts briefly with the salt solutions, then carefully replacing the salt by sorbitol to maintain compaction. Subsequent flotation in a sucrose solution then produced a

much lesser degree of disturbance. However some degree of aggregate dispersion appeared to be essential. Prolonged protoplast compaction following salt treatment eventually lead to lysis even though the salt may have been replaced.

Protoplast aggregates which survived flotation were eventually transferred to a culture medium and maintained under sterile conditions for periods of up to 72 h. Very few aggregates showed any sign of rounding-off. Feulgen staining was used to detect any multinucleate fusion body formation. Some indication of multinucleation was observed in sodium nitrate treated material but it is considered that the occurrence was not sufficiently high to be statistically significant despite controls to reduce spontaneous fusion.

Visually distinguishable protoplasts (see Section 4.3.2) were used in all of the tests but it was found that the protoplasts tended to form aggregates of like types and the only aggregates seen to form rounded-off fusion bodies were those of tobacco mesophyll protoplasts alone. The different flotation properties of some of the protoplasts were thought to contribute to this effect but do not entirely explain it.

The various salt treatments are analysed in Table 5.1. Based on the effects described above, the following specific effects were scored according to intensity.

- a. protoplast adhesion upon sinking
- b. protoplast adhesion upon centrifugation (150 x g for 2 min)
- c. adhesion maintained after 5 min salt treatment followed by flotation in sucrose
- d. adhesion maintained after 30 min salt treatment (or 5 min salt treatment followed by 30 min in sorbitol to maintain compaction) followed by flotation in sucrose.

- e. protoplast aggregates survive 24 h in culture
- f. protoplast aggregates survive more than 24 h in culture
- g. rounding-off and multinucleation observed
- h. protoplasts damaged by brief salt treatment
- i. protoplasts damaged by prolonged salt treatment
- j. "halo" observed around some protoplasts
- k. indirect protoplast adhesion

Table 5.1 Protoplast response to inorganic salt treatment

Treatment		Response										
		a	b	c	d	e	f	g	h	i	j	k
Centrifuged control (5min, 150 x g)		+	+	-iv	-iv							
Salt	Molarity											
Sodium nitrate	0.61	++	++	+	++	-	-	-	-	++	+	+
	0.45	+++	+++	++	+++	++	+	+	-	+	-	+
	0.3	+++	+++	+	+++	++	+	+	-	+	-	-
	0.2	++	++	+	++	+	-	-	-	-	-	-
	0.15	++	++	+	++	-	-	-	-	-	-	-
	0.1	+	+	-	+	-	-	-	-	-	-	-
	0.05	-	+	-	-	-	-	-	-	-	-	-
sodium nitrite	i	++	++	+	++	+	-	-	+	++	+++	++
	ii	+++	+++	+	+++	+	-	-	+	++	++	++
sodium chloride	i	++	+++	+	+++	-	-	-	-	++	+	+
	ii	++	+++	+	+++	+	-	-	-	+	-	-
potassium nitrate	i	++	++	+	++	-	-	-	+	++	+++	+++
	ii	+++	+++	++	+++	-	-	-	+	++	++	++
potassium chloride	i	++	++	+	++	-	-	-	-	++	+	+
	ii	++	+++	+	+++	+	-	-	-	-	-	-
calcium nitrate	i	+	++	+	+	-	-	-	+	+++	++	++
	ii	++	+++	+	++	+	-	-	+	++	+	+
calcium chloride	i	+	++	+	++	-	-	-	+	++	+	+
	ii	+	+++	+	+++	+	-	-	-	+	-	-
ammonium nitrate	i	+	++	-	++	-	-	-	++	+++	+	+
	ii	+	++	-	++	-	-	-	++	+++	+	+
ammonium chloride	i	++	++	+	++	-	-	-	-	++	+	+
	ii	++	++	+	++	-	-	-	-	+++	-	-
ammonium sulphate	i	+	++	+	++	-	-	-	+	++	-	+
	ii	+	++	+	++	-	-	-	-	+	-	-
lithium chloride ^v	i	++	+++	+	+++							
barium chloride	i	+	++	+	+++							
magnesium chloride	i	++	++	+	++							
aluminium chloride	i	++	++	+	+++							
sodium nitrate/calcium nitrate	iii	++	+++	+	++							
sodium chloride/aluminium chloride	iii	++	+++	+	+++							

- Notes:
- i. osmosity equivalent to 0.77 M sucrose
 - ii. molarity = $i \times \frac{1}{2}$
 - iii. 1/1, v/v mixtures, osmosity equivalent to 0.77M sucrose
 - iv. flotation followed centrifugation, no salt treatment
 - v. lithium chloride and following treatments, cursory study only

The amount of information which can be obtained from light microscopic observations is obviously limited. Electron microscopic observations provide more detailed information.

5.1.3 Individual salt effects, electron microscopic observations

The most obvious result of salt treatment is protoplast adhesion. This was occasionally observed in untreated material. Figure 5.1 shows three small subprotoplasts or cytoplasmic fragments formed during the plasmolysis of tobacco mesophyll tissue. The plasmalemmae are closely adpressed producing a 5-layered effect from the two 3-layered membranes. A region of adhesion between a petunia mesophyll and a tobacco mesophyll protoplast is shown in Figure 5.2. The adhesion, a rare occurrence, was produced by centrifugation. Both plasmalemmae can be distinguished here and there is a gap of some 15nm between them although they run exactly parallel. No observations were made to suggest that such adhesion progressed to fusion.

Following salt treatment, protoplast adhesion on a much larger scale was observed. Using protoplasts of different origins, intraspecific, interspecific and intervarietal adhesion was demonstrated. Figure 5.3 shows adhesion between a tobacco, Xanthi mesophyll and a tobacco, Xanthi, epidermal protoplast whilst in Figure 5.4a, a similar degree of adhesion is observed between tobacco, Xanthi, and tobacco, Bright Yellows, mesophyll protoplasts. The latter protoplasts are clearly distinguishable, but in some cases, Bright Yellows protoplasts were further identified by the presence of TMV particles. (Fig. 5.4b).

A tobacco Xanthi epidermal protoplast and a petunia mesophyll protoplast adhere, not by direct membrane contact as in the above examples but by cytoplasmic protrusions. (Fig. 5.5). Both types of

30

Membrane adhesion

Figure 5.1 Three membrane bound cytoplasmic fragments adhere together. At the points of adhesion, the two three-layered membranes appear as a single five-layered membrane.

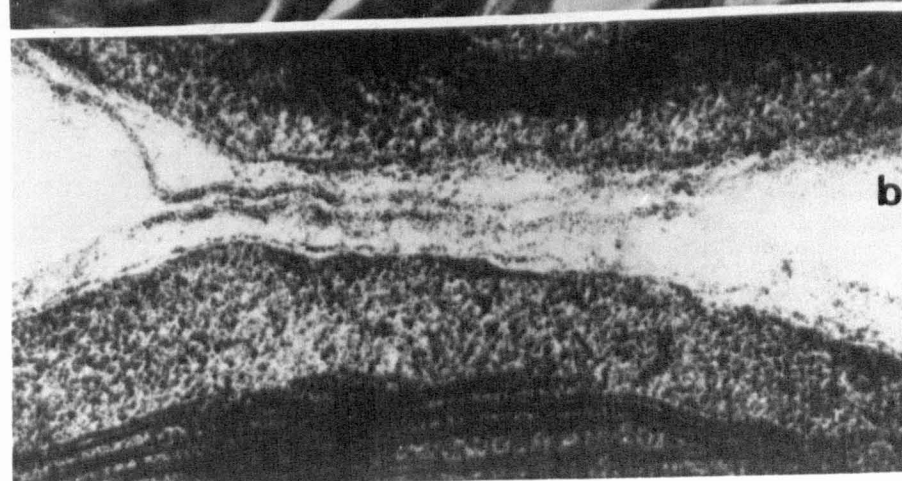
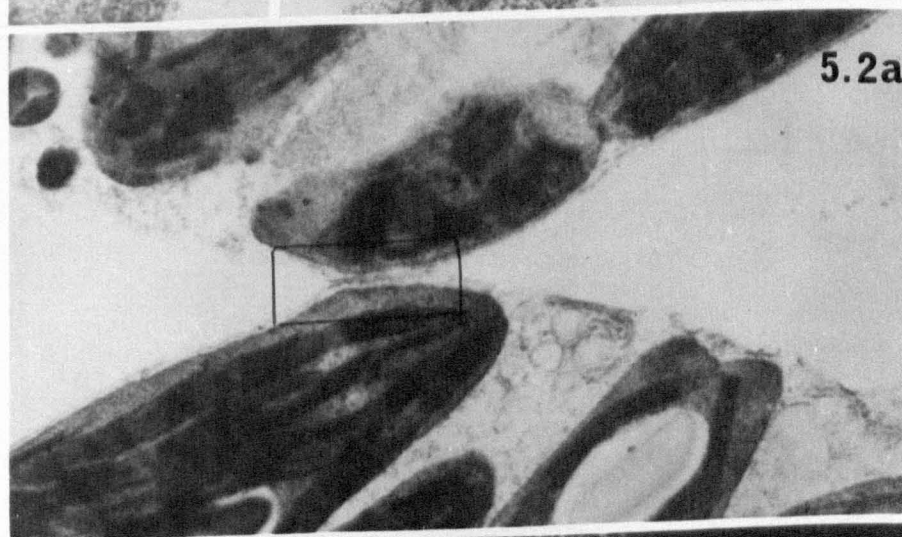
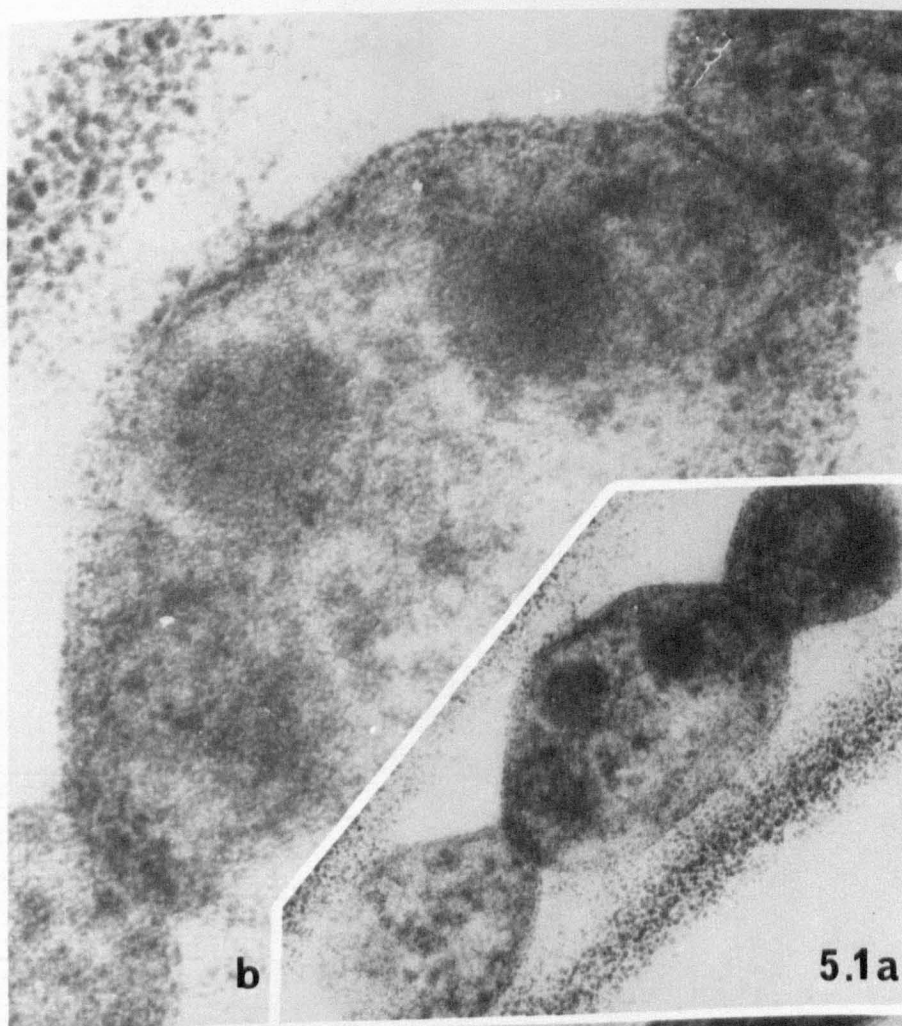
Figure 5.1a x 120,000

Figure 5.1b x 350,000

Figure 5.2 A tobacco mesophyll(lower) and a petunia mesophyll(upper) protoplast are adhering together after centrifugation. The plasmalemmae run parallel but are separated by approximately 15nm.

Figure 5.2a x 20,000

Figure 5.2b x 100,000



Mixed protoplast adhesion (salt induced)

Figure 5.3 A tobacco (Xanthi) mesophyll (upper) and tobacco (Xanthi) epidermal (lower) protoplast are adhering.

x 4,000

Figure 5.4a A tobacco (Bright Yellows) mesophyll(left) and
a tobacco(Xanthi) mesophyll protoplast(right)
are adhering. x 6,000

x 6,000

Figure 5.4b Detail of TMV particles in a tobacco (Bright Yellows)
protoplast. x 15,000

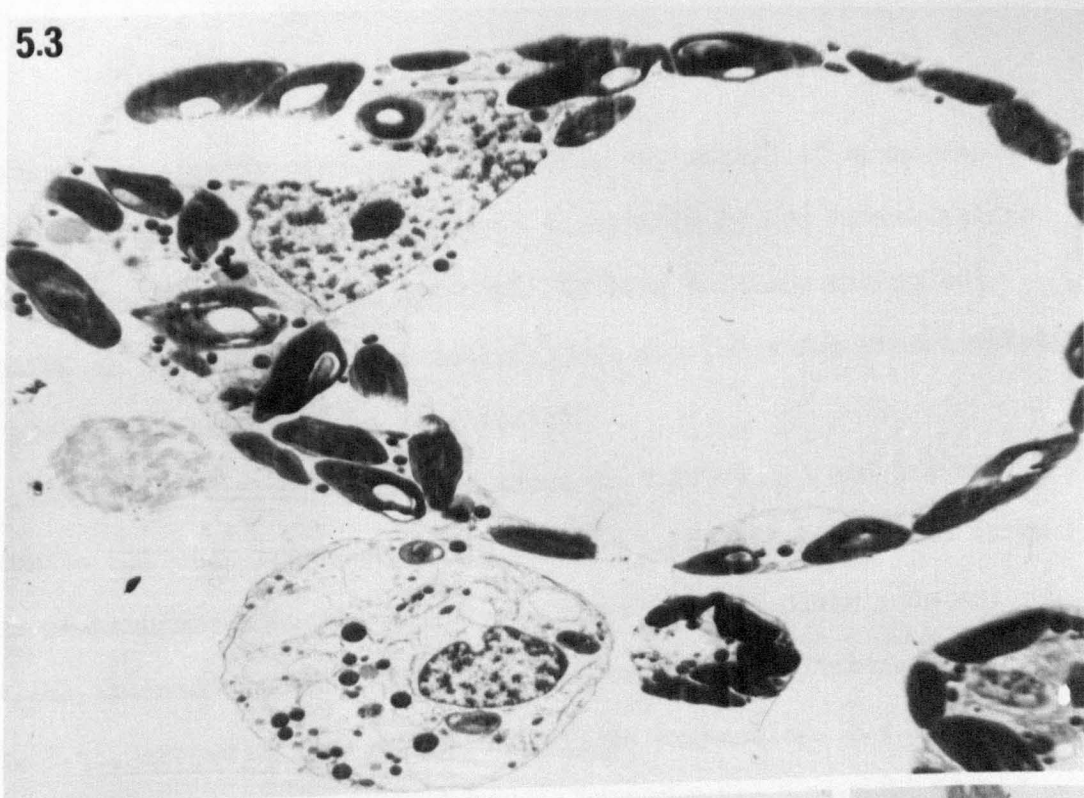
x 15,000

Figures 5.5 a and b A tobacco (Xanthi) epidermal (upper) and
petunia mesophyll protoplast are adhering. The adhesion
is by plasmalemmar protrusions. (Figure 5.5 b)

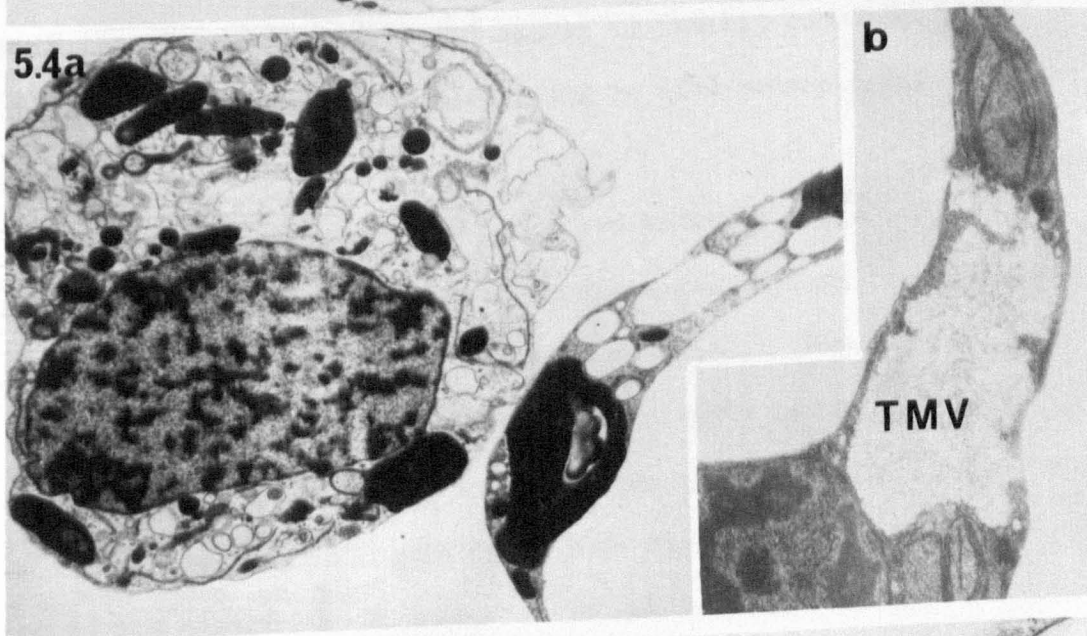
Figure 5.5a x 3,250

Figure 5.5b x 20,000

5.3



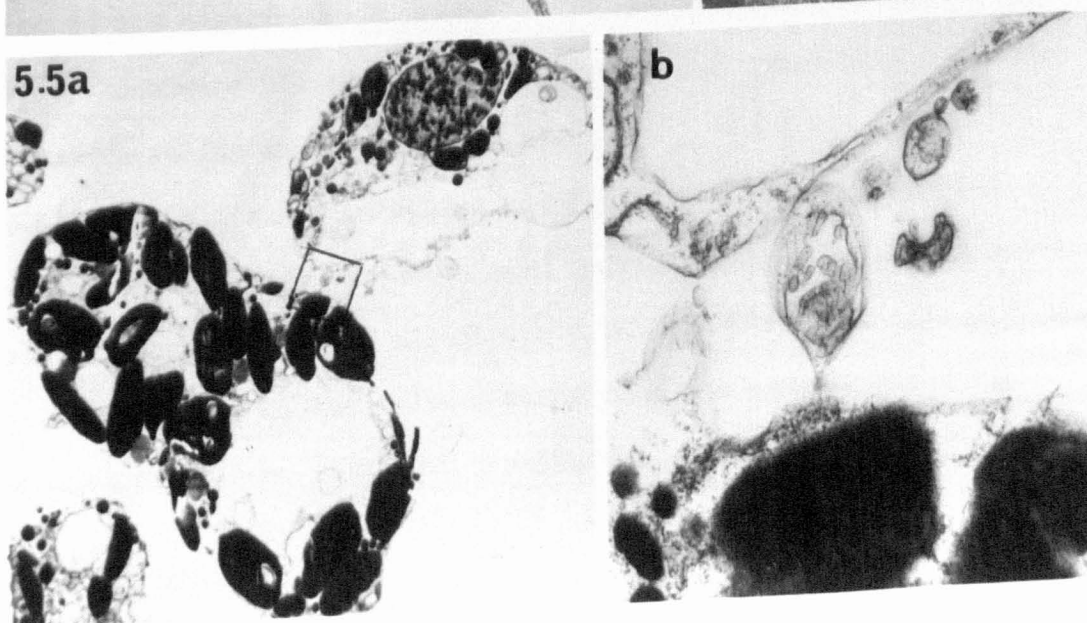
5.4a



b

TMV

5.5a



b

adhesion are clearly distinguishable from any stages of spontaneous fusion. It was therefore considered acceptable to use intraspecific tobacco, Xanthi, or petunia mesophyll systems to study protoplast adhesion in depth, since more material was available for experimentation and protoplast handling was facilitated.

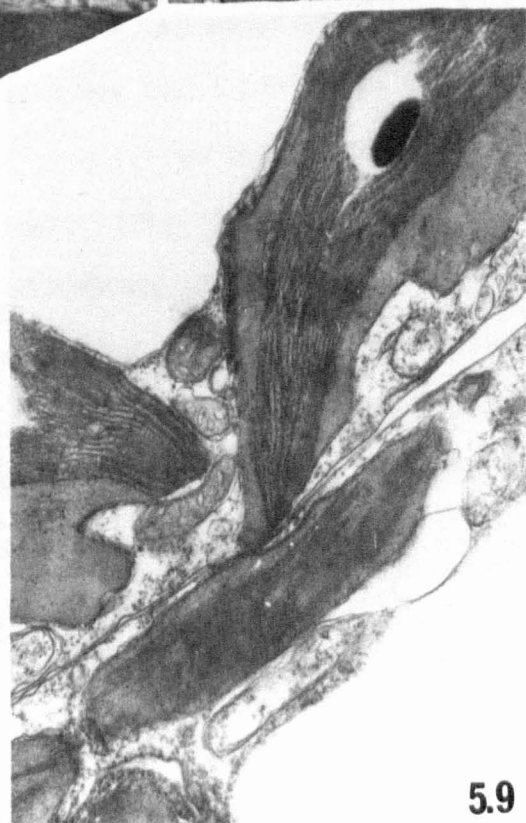
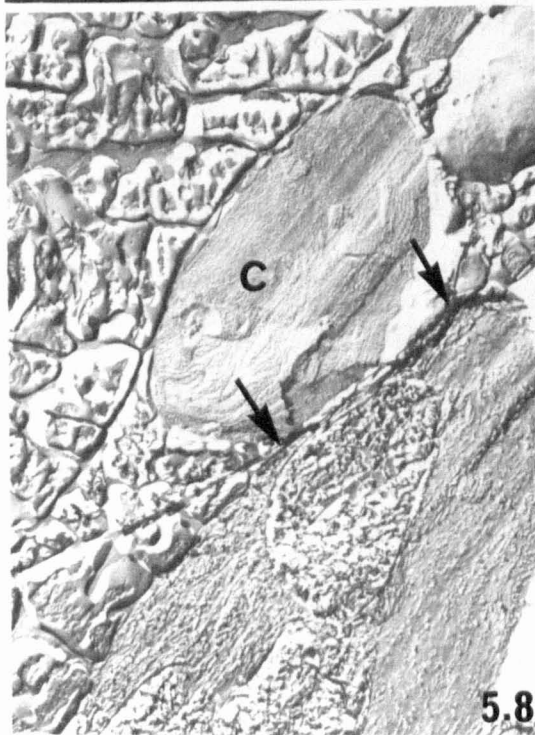
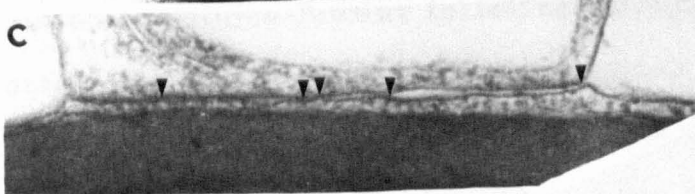
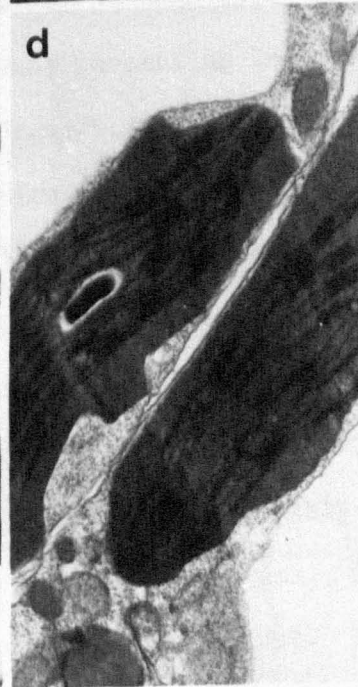
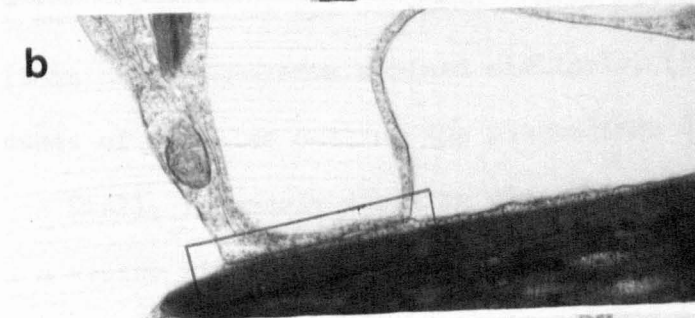
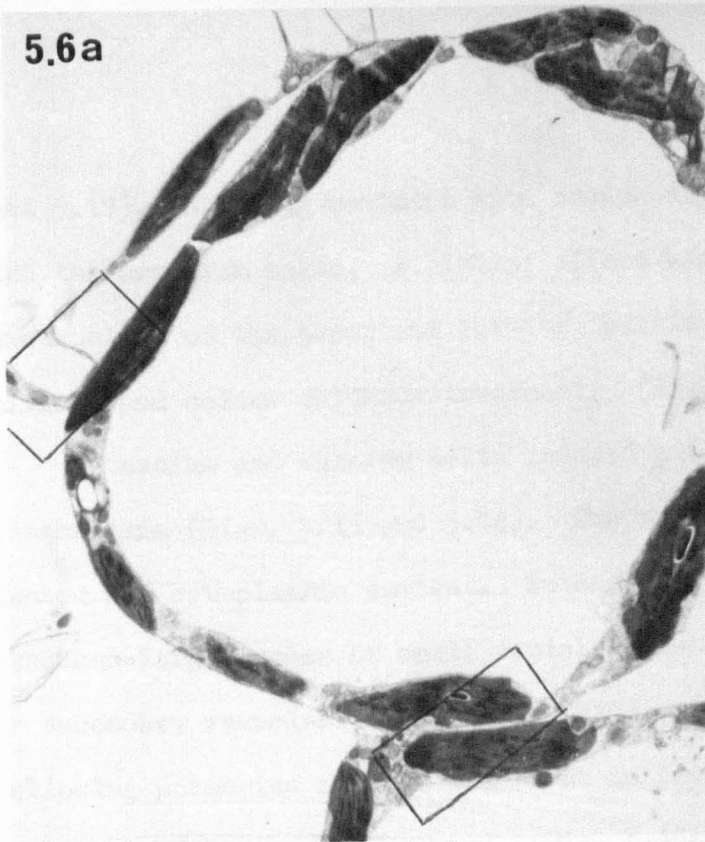
The direct membrane adhesion shown in Figures 5.3 and 5.4 was common to all salt treatments. In Figures 5.6a to 5.6d, fairly large areas of membrane adhesion are seen. The membranes clearly do not run parallel, points of much closer adhesion occurring at intervals. (Fig. 5.6c, arrows). The presence of large organelles, e.g. chloroplasts beneath the plasmalemmae does not appear to inhibit adhesion. Frequently cytoplasmic debris was observed adhering to live protoplasts. (Fig. 5.7).

Frozen etched preparations of unfixed material (Fig. 5.8) showed membrane adhesion without the accompanying distortion of the protoplast outline common in thin sections of fixed material. (Fig. 5.6b). This may be artifactual but the adhesion itself clearly is not. To aid interpretation an area similar to that shown in the replica (Fig. 5.8) is shown in thin section in Figure 5.9. Both Figures show a plane at right angles to the adhering membranes. It is interesting to note that although many replicas were prepared, none demonstrated a tangential membrane fracture revealing an adhering surface.

Although all salts induced a similar type of direct membrane adhesion, other effects showed a much greater diversity. The formation of plasmalemma protrusions was observed in protoplasts treated with high molarities of most of the salts. These took the form of drawn out strands of cytoplasm, with some pinching off of small spheres, (Figs. 5.10

Salt induced protoplast adhesion

- Figure 5.6a Three adhering protoplasts. x 6,000
- Figures 5.6b to d Details of the above figure. The plasmalemma adhesion is stronger at certain points. (Figure 5.6c arrows).
Figure 5.6b x 20,000
Figure 5.6c x 50,000
Figure 5.6d x 17,500
- Figure 5.7 A broken down chloroplast adhering strongly to a live protoplast. x 11,000
- Figure 5.8 A freeze-etch replica of an adhesion region. The adhering membrane is arrowed. (C=chloroplast)
x 20,000
- Figure 5.9 An equivalent region to the above figure, shown in thin section. x 38,000



and 5.11), following treatment with sodium nitrate, chloride and nitrite and the ammonium salts. A similar effect was produced in the vacuole, protrusions of the tonoplast forming, particularly following potassium nitrate and sodium chloride treatment. (Fig. 5.12).

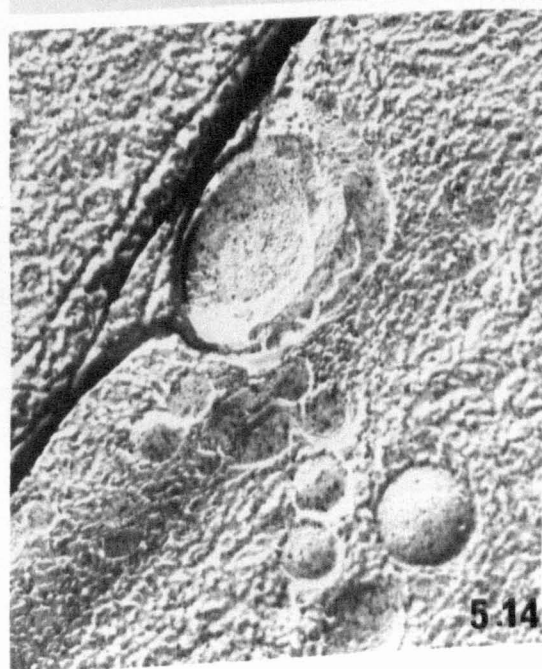
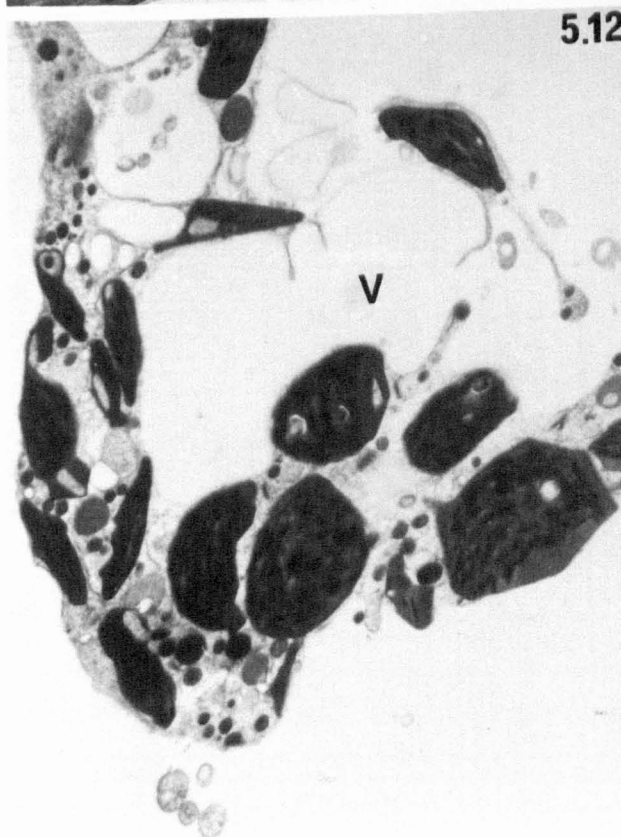
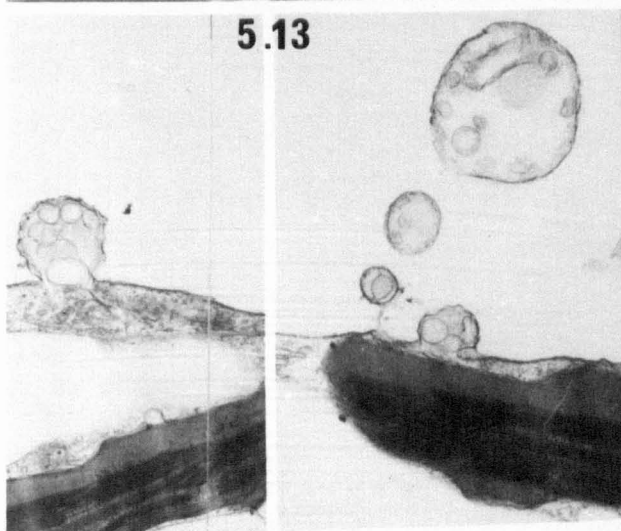
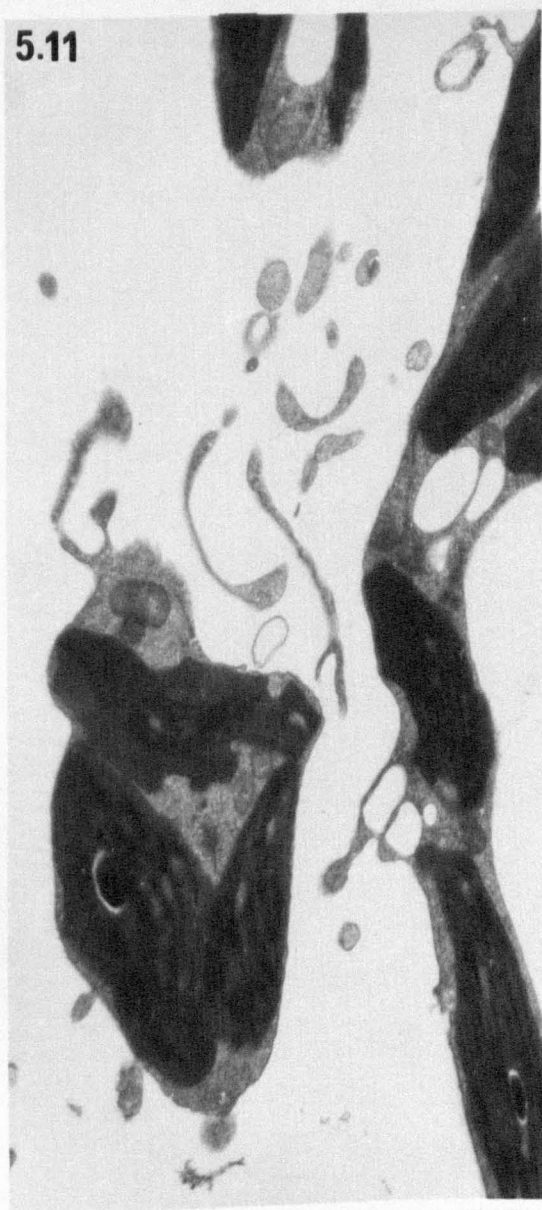
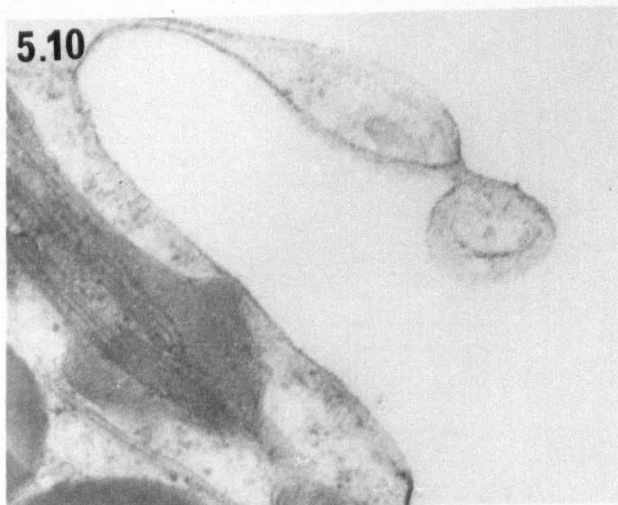
Potassium and calcium salts induced a fine "bubbling" of the plasmalemma (Figs. 5.13 and 5.14). The small spheres of membrane lacked any cytoplasmic content. Intense activity of this type produced large masses of small vesicles somewhat resembling lomasomes or secondary vacuoles. (Mahlberg, 1972). (Figs. 5.15 and 5.16). Following potassium nitrate treatment in particular, an intense staining of the plasmalemma was observed. (Fig. 5.15a). Small vesicles lying within the plasmalemma stained similarly, (Fig. 5.17) although the masses of vesicles outside the plasmalemma did not.

Small, intensely staining plasmalemmar projections were observed in a sodium nitrate treated preparation (Fig. 5.18), with possibly related structures forming following calcium chloride treatment. (Figure 5.19) The latter salt specifically induced an unusual plasmalemmar effect. The membrane appeared to break and reseal with the underlying outer membrane of the chloroplast. (Figs. 5.20a and 5.20b). An interesting observation in the latter figure is that of two bilayered membranes apparently joining to form one bilayer. (Fig. 5.20b, arrow and inset).

Returning to the more common effects of plasmalemmar protrusions and bubble formation. These were frequently involved in protoplast adhesion. (Figs. 5.21 and 5.24). The total area of membrane involved in the adhesion was often considerable. The protrusions facilitated membrane contact in regions where direct adhesion would have involved

Effects induced by salt treatment

- Figure 5.10 Plasmalemmar protrusions induced by sodium chloride treatment. x 45,000
- Figure 5.11 Two protoplasts, the left one cut tangentially, showing sodium nitrate induced protrusions. x 20,000
- Figure 5.12 A protoplast with both plasmalemma and tonoplast protrusions produced by potassium nitrate treatment. (v=vacuole) x 10,000
- Figure 5.13 Two regions of a protoplast plasmalemma showing 'bubbling' induced by potassium nitrate treatment. x 20,000
- Figure 5.14 A freeze-etch replica showing plasmalemma 'bubbling' after potassium nitrate treatment. x 60,000



Effects induced by salt treatment

Figures 5.15a and b A protoplast treated with potassium nitrate.

The plasmalemma is darkly stained, as are underlying vesicles. A region of plasmalemmar 'bubbling' is only lightly stained (Fig 5.15b)

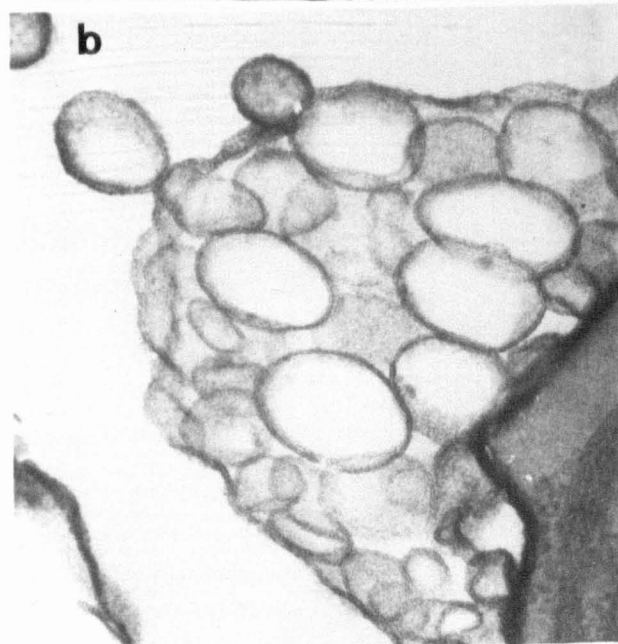
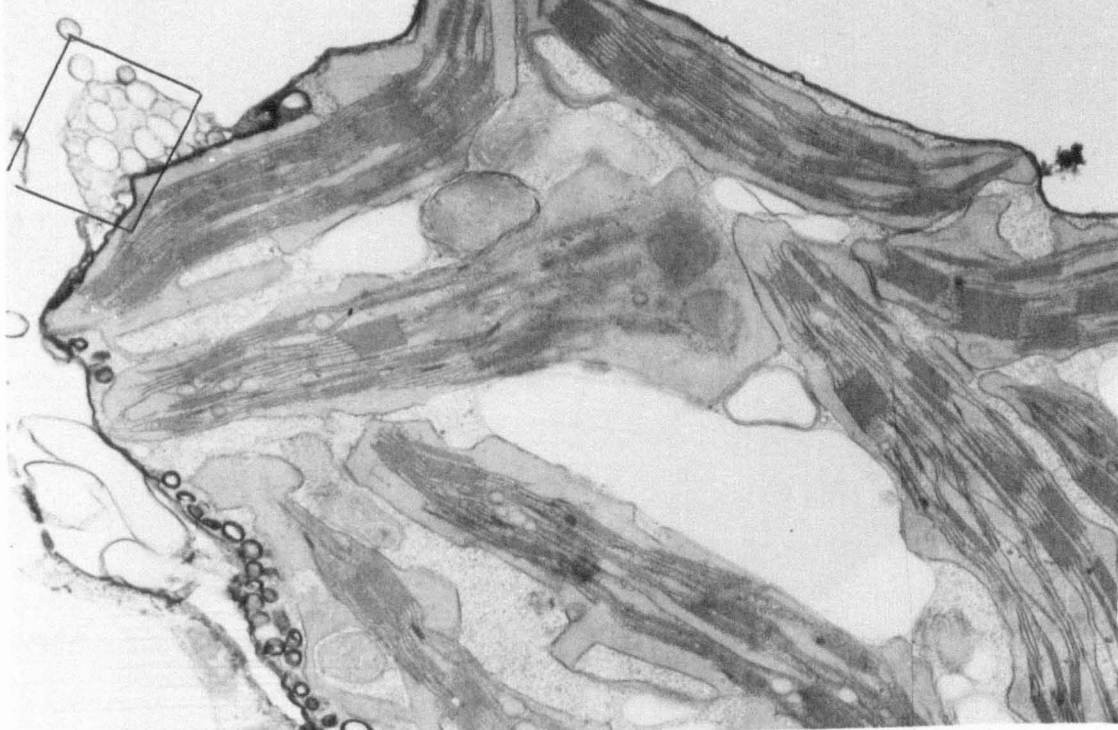
Figure 5.15a x 22,500

Figure 5.15b x 90,000

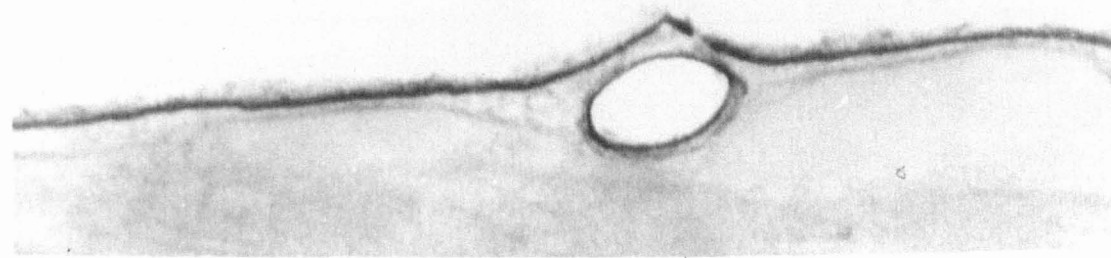
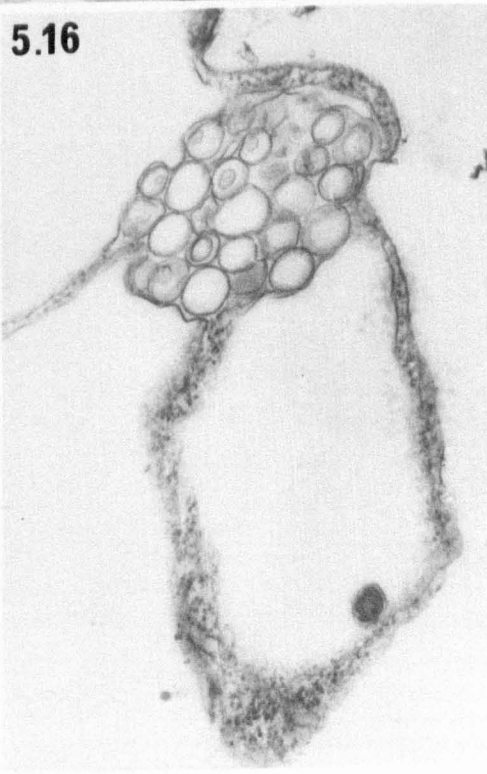
Figure 5.16 A region of plasmalemmar 'bubbling' induced by calcium chloride treatment. x 30,000

Figure 5.17 A detail of plasmalemma and vesicle staining in
a potassium nitrate treated protoplast.
x 100,000

5.15a



5.17



Effects induced by salt treatment

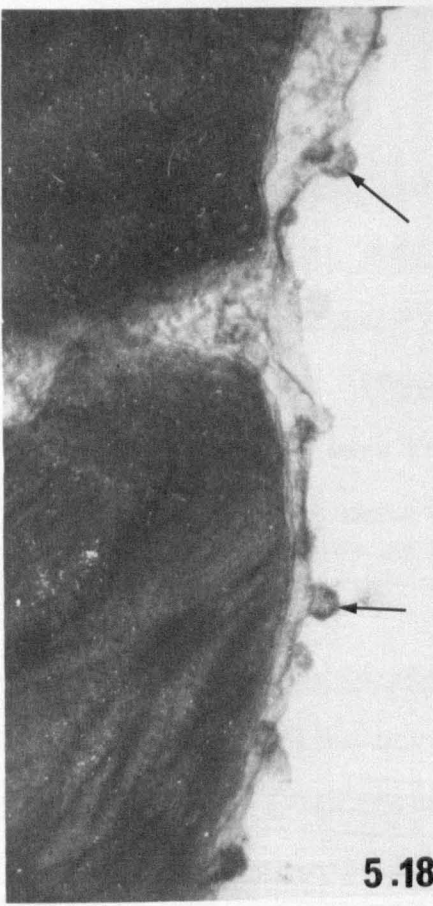
Figure 5.18 The plasmalemma of a sodium nitrate treated protoplast showing projecting regions of intense staining.
(arrowed). x 65,000

Figure 5.19 The plasmalemma of a calcium chloride treated protoplast. Dense membrane figures are seen(arrowed).
x 22,500

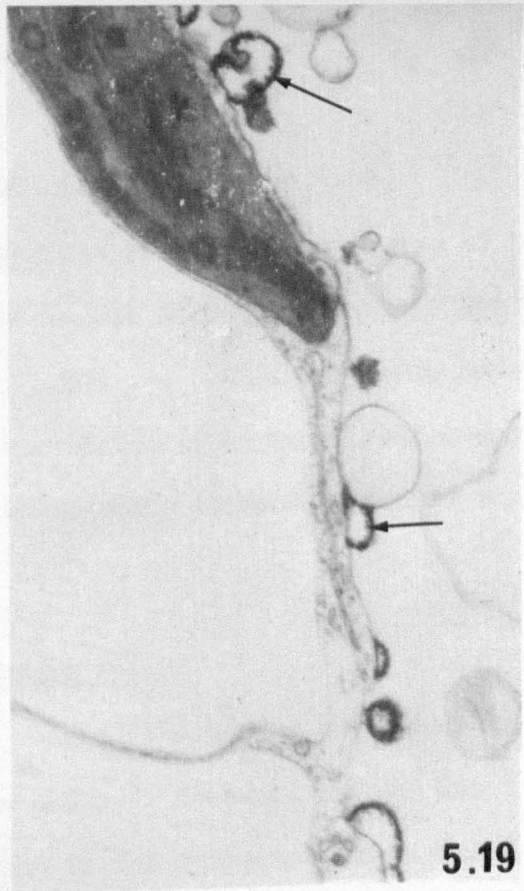
Figures 5.20a and b Two protoplasts adhering after calcium chloride treatment. The plasmalemma of the protoplast to the left has apparently broken and resealed to the outer chloroplast membrane.(arrows) In one region, the two membranes appear to form a single bilayer.(inset.)

Figure 5.20a x 55,000

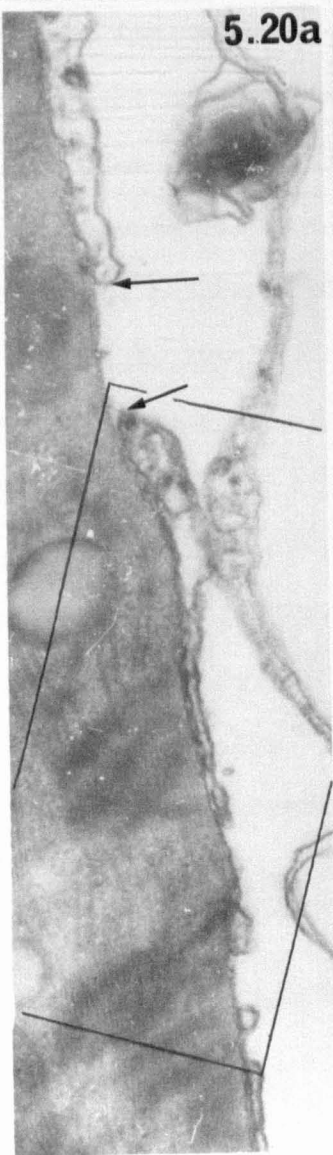
Figure 5.20b x 110,000



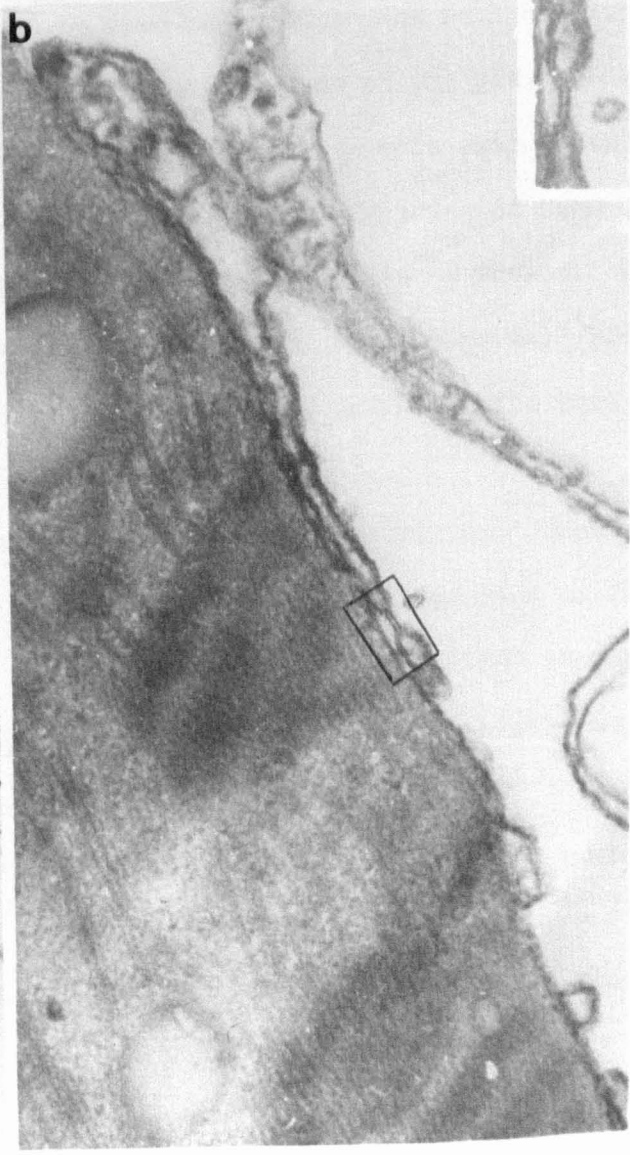
5.18



5.19



5.20a b



extreme distortion of the protoplasts. (Figs. 5.22 and 5.23).

It was often difficult in such regions to follow the course of the individual protoplast plasmalemmae and it was therefore possible that membrane fusion had occurred. (Figs. 5.24a and 5.25). However, no unequivocal examples of fusion were seen. Definite membrane fusion was only observed in regions of direct membrane contact, induced by sodium nitrate treatment.

5.1.4 Sodium nitrate induced membrane fusion.

Frequent observations were made of the plasma membranes showing an apparent continuity. (Figs. 5.26a and b.) However, due to the orientation of the section with respect to the membranes, such observations were considered too ambiguous to be used as evidence for membrane fusion. In contrast, sections cut perpendicularly to the plane of the plasmalemmae, give clear evidence for fusion. (Figs. 5.27a and b). In this example the discontinuous membranes run more or less parallel at a spacing of some 250 nm. The breaks in the membranes are considered to be points of fusion. (Fig. 5.27b, arrows). No intermediate stages of fusion were observed. Note that there are no e.r. membranes in the fusion region, unlike in the early stages of spontaneous fusion.

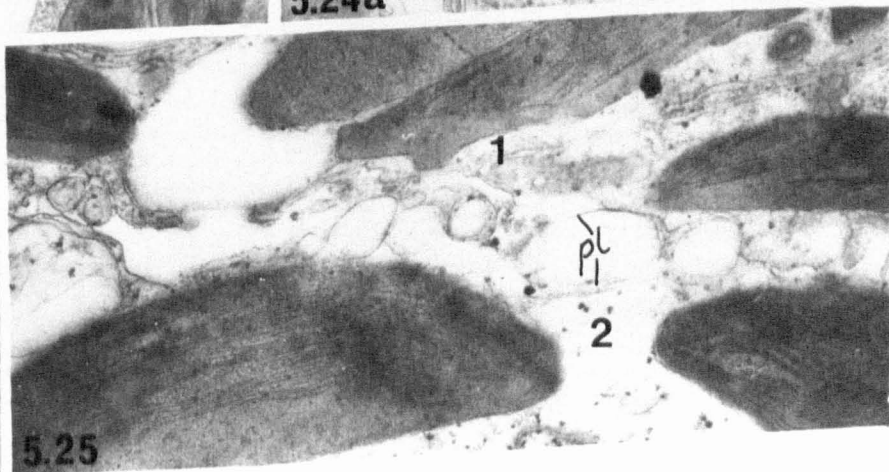
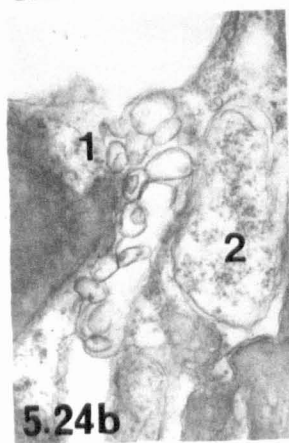
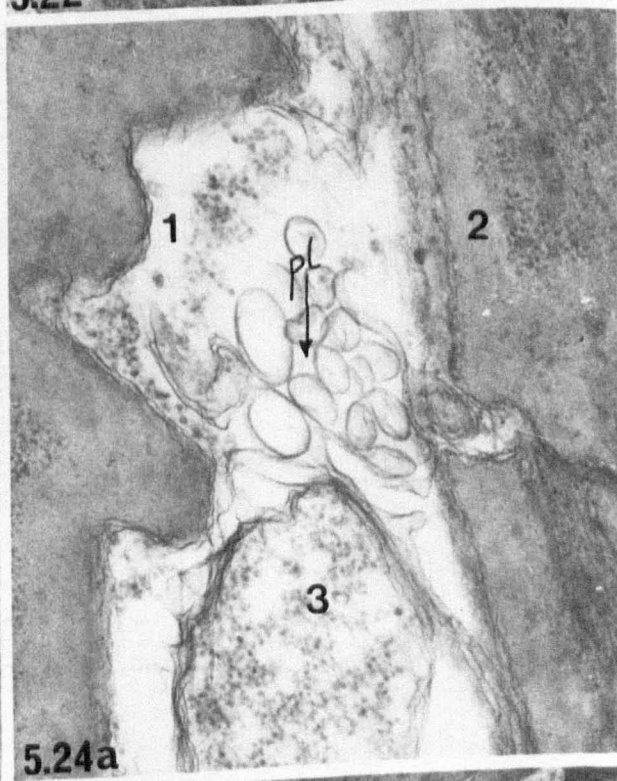
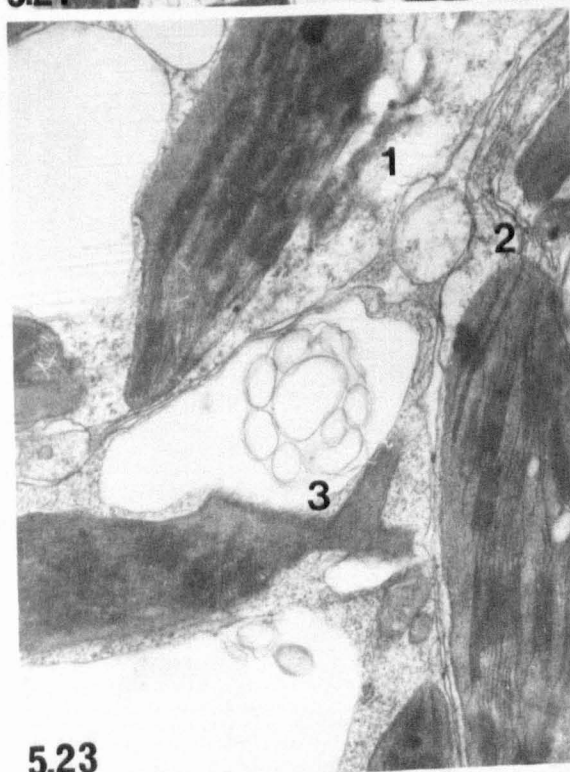
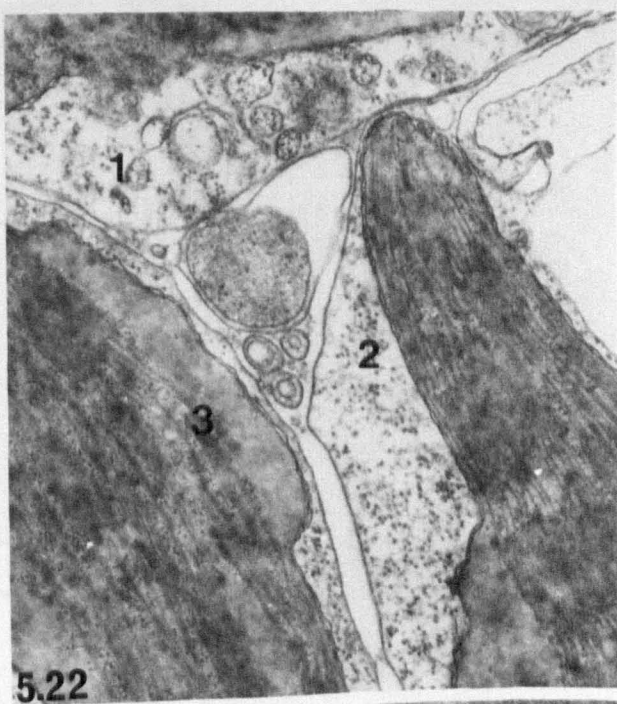
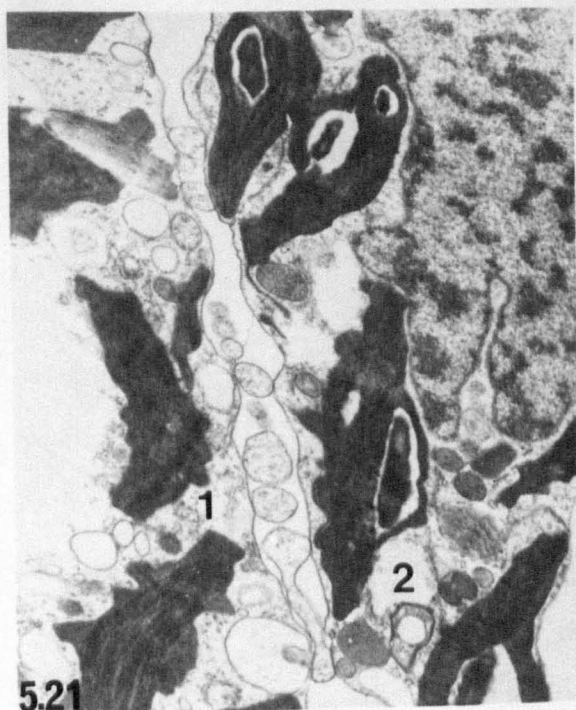
Figure 5.28 shows a similar but more advanced situation. Membrane fusion has progressed so that there is a much higher proportion of fused to unfused membrane. Part of a large fusion body (see legend) is shown in Figures 5.29a and 5.29b. The protoplasts have fused in areas marked by the concentration of organelles, including the nuclei. Few membrane fragments remain, separating the cytoplasms. (Figs. 5.29a, arrows and 5.29b).

Successful fusion, confirmed and clarified by electron microscopy,

Effects induced by salt treatment

(Individual protoplasts are numbered. pl = plasmalemma)

- Figure 5.21 Cytoplasmic protrusions lie between two adhering,
sodium nitrate treated, protoplasts. x 12,000
- Figure 5.22 Protrusions lie in the triangular space between
three adhering, sodium nitrate treated, protoplasts.
x 30,000
- Figure 5.23 Membrane 'bubbles' lie in an apparent vesicle in one
of three adhering, ammonium nitrate treated, protoplasts.
It is probably, in fact, a transversely cut depression
in the plasmalemma. x 30,000
- Figure 5.24a Membrane 'bubbles' lie between adhering, sodium
chloride treated, protoplasts. It is difficult to
identify the protoplast plasmalemmae. x 50,000
- Figure 5.24b Again, sodium chloride treated protoplasts. Here
the plasmalemmae are somewhat clearer. x 30,000
- Figure 5.25 The membrane 'bubbles' between the protoplasts may
have fused with the plasmalemmae of the potassium
nitrate treated protoplasts. x 22,500



Sodium nitrate induced membrane fusion

Figures 5.26a and b Two protoplasts are adhering and possibly fusing. Since the plasmalemmae are not cut perpendicularly, the image is ambiguous.

Figure 5.26a x 7,000

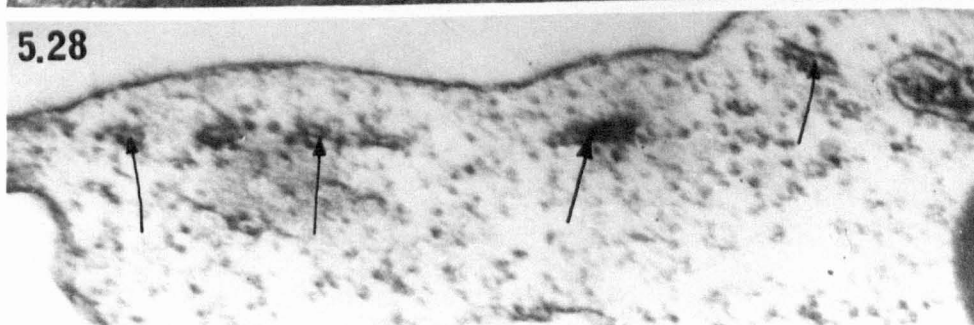
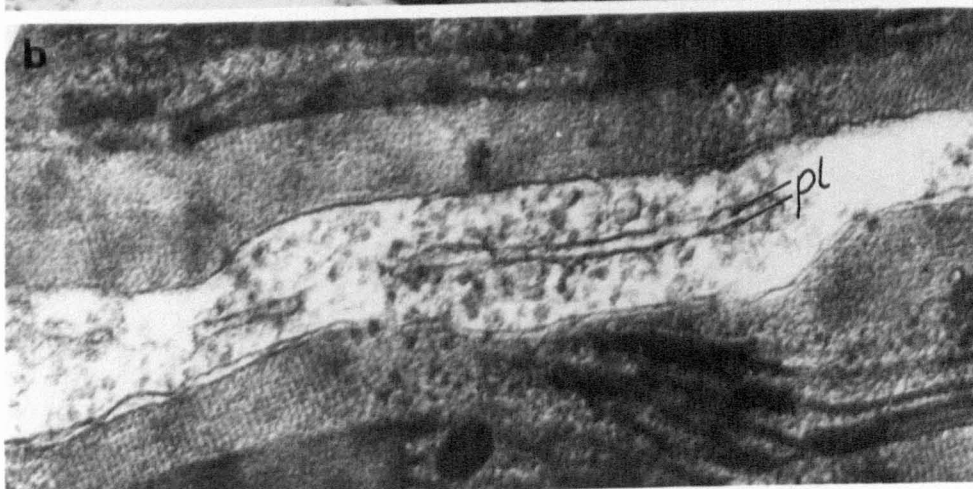
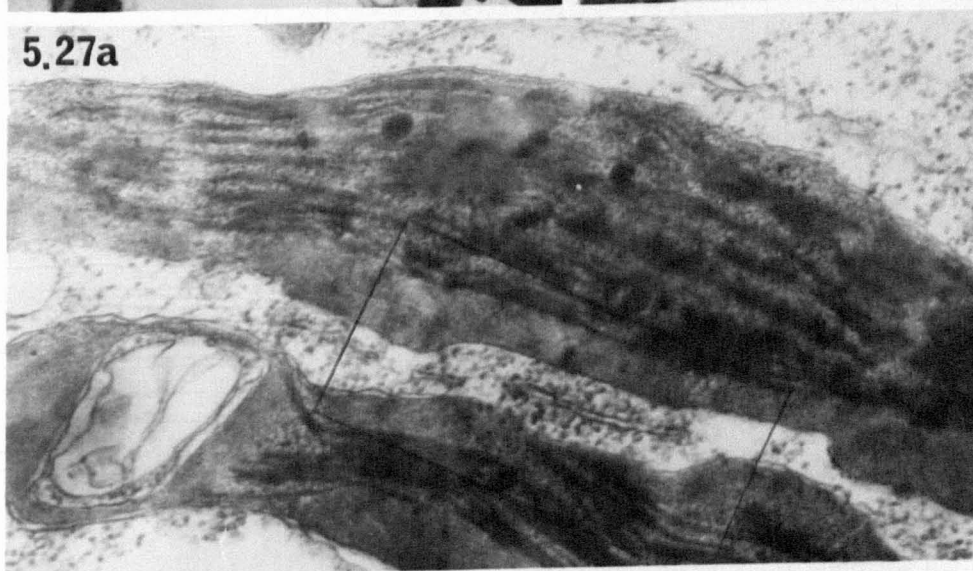
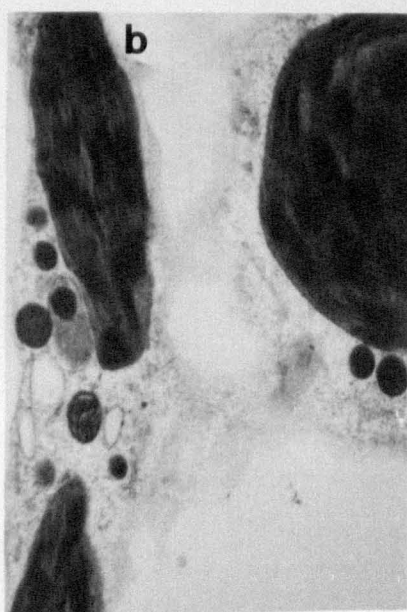
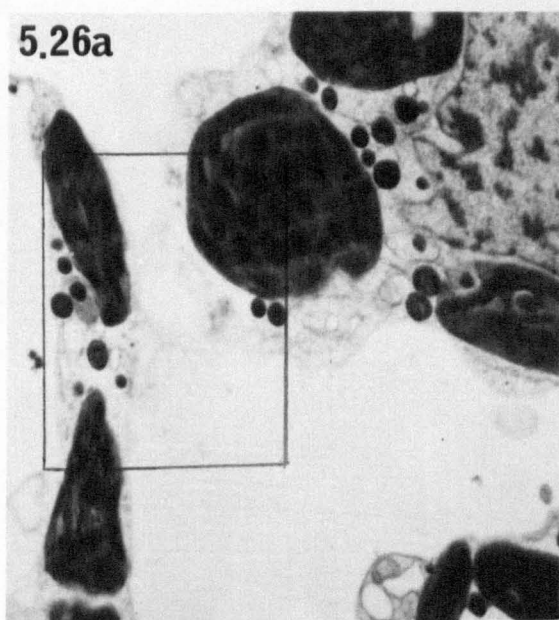
Figure 5.26b x 12,000

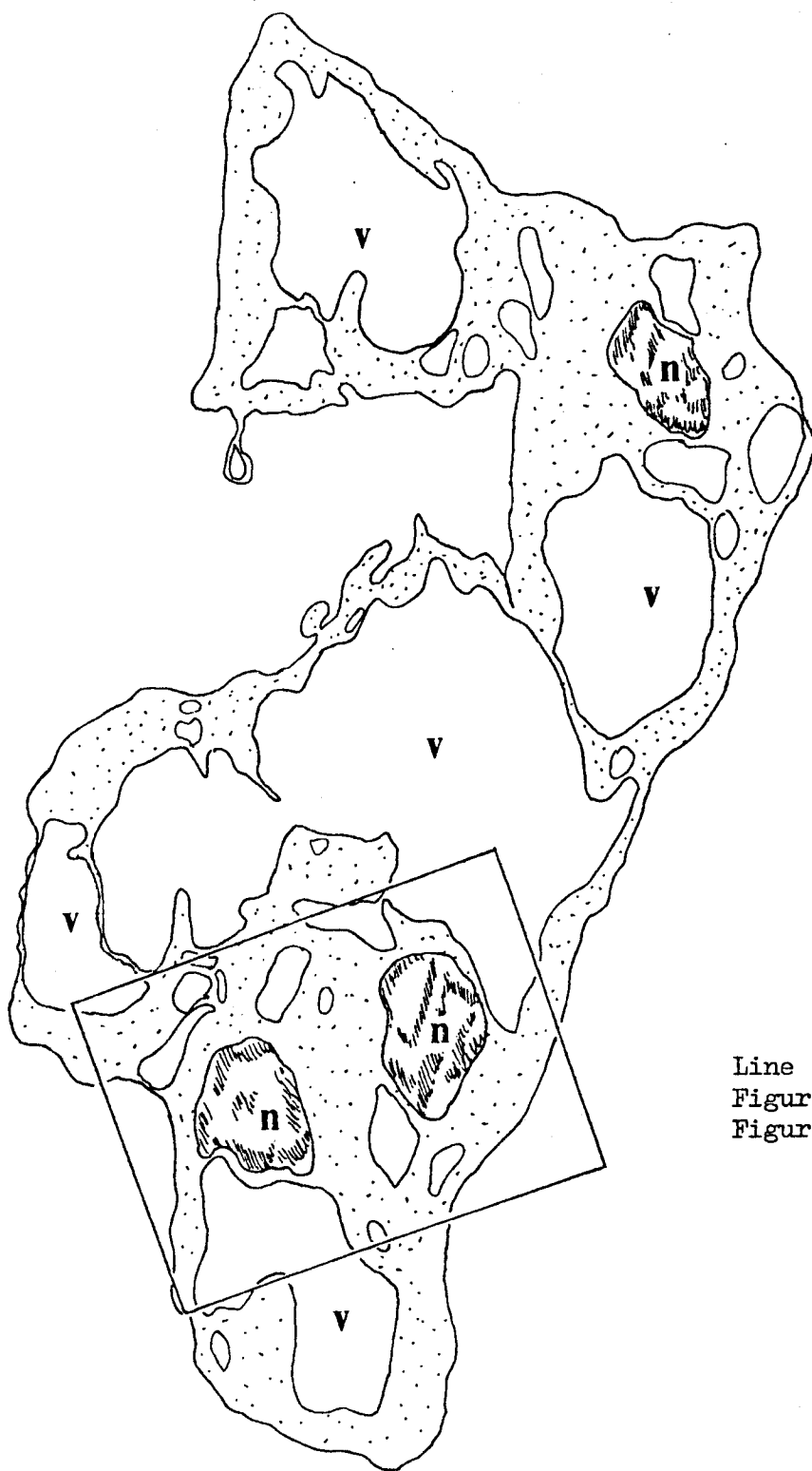
Figures 5.27a and b A section through a fusion region. The adhering protoplast plasmalemmae remain in part, (pl) broken by the points of fusion.

Figure 5.27a x 55,000

Figure 5.27b x 120,000

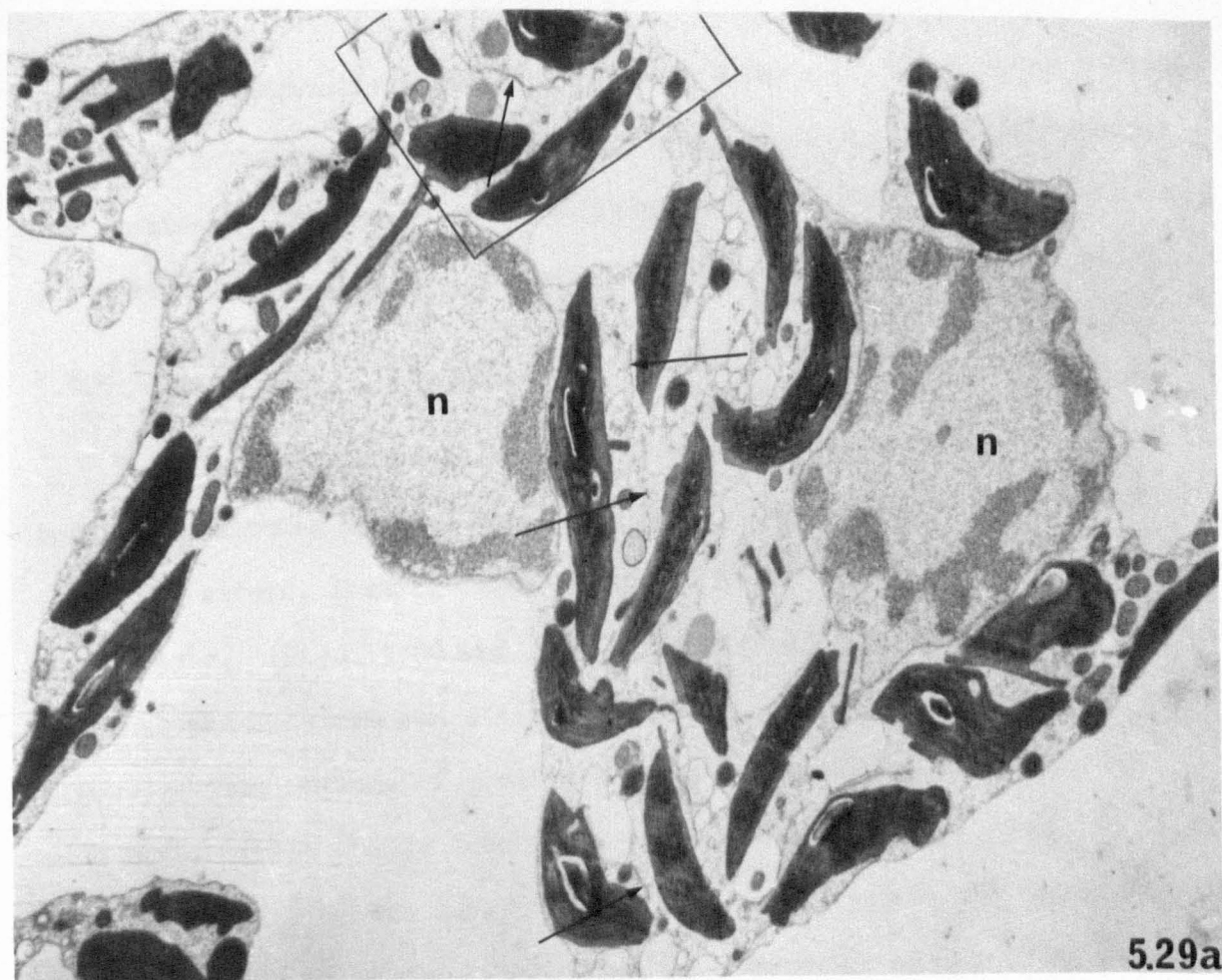
Figure 5.28 A more advanced stage than the above. Less of the adhering plasmalemma is visible, and there are no large organelles in the region. The remaining plasmalemma is arrowed. x 100,000





Line drawing x 2,500
 Figure 5.29a x 6,500
 Figure 5.29b x 25,000

Figure 5.29 A sodium nitrate induced fusion body. The region shown in Figure 5.29a is boxed in the drawing above. The nuclei (n) of adjacent protoplasts are separated by a broad area of cytoplasm. A few remnants of the fused plasmalemmae can be traced. (Fig. 5.29a arrows and Fig. 5.29b). The region shown in Figure 5.29b is boxed in Figure 5.29a. The vacuoles (v) of the original protoplasts remain separate.



was only unambiguously observed in sodium nitrate (0.4M) treated tobacco mesophyll protoplast preparations. This supports the light microscopic observations. (Section 5.1.2) No intervarietal or interspecific fusion was observed, nor definite intraspecific fusion induced by other salts.

5.1.5 Post-fusion events

Twenty-four hours after sodium nitrate treatment, the fusion bodies are showing signs of rounding-off. The origin^{al} areas of fusion appear to extend, forming large flattened faces of contact between the protoplasts. (Figs. 5.30 and 5.31). Frequently remnants of the original plasmalemmae can still be identified. (Figs. 5.30 b and c arrows). Some regions of plasmalemma remain separated by relatively wide gaps. (Figs. 5.30c and 5.31b, double arrows).

A migration of the larger organelles, mitochondria and chloroplasts, appears to be taking place, resulting in a thinning of the cytoplasm in the central fusion regions, (Fig. 5.31a, arrows) and a corresponding crowding of organelles on or near the periphery of the fusion body. (Figs. 5.30a and 5.31a, double arrows). There are breaks in the continuity of organelle distribution, marked by a proliferation of small vesicles and an absence of chloroplasts, on the fusion body periphery at the junctions of the contributing cytoplasmic masses. (Fig. 5.30a, arrows).

Over the next 12 h (total time after sodium nitrate treatment, 36 h) further organelle redistribution occurs. In some regions, the vacuoles of adjacent fusing protoplasts remain separated only by the two tonoplasts. (Fig. 5.32b). In Figure 5.33 this strand appears to be under tension and on the point of breaking. As can be seen in Figures 5.32a and 5.33a, the changes at the protoplast interfaces do not occur synchronously.

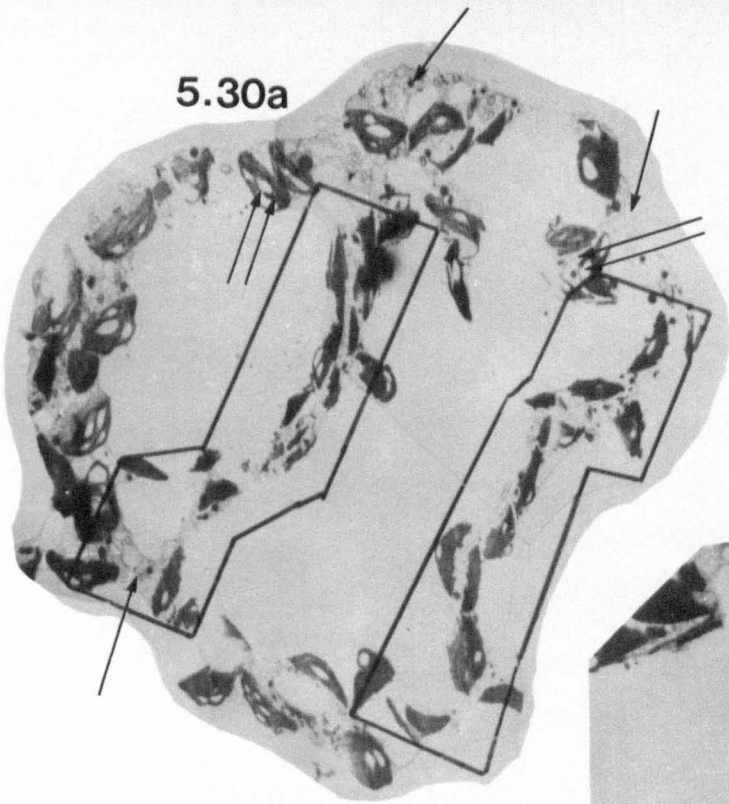
Post fusion events

Figures 5.30a to c A region of a large induced fusion body, showing the fusion between three protoplasts. The fusion interfaces are enlarged in Figures 5.30b and c. Remnants of plasmalemma can be seen in the fusion regions, some are adhering closely (pl) others are separated by wide gaps (Figure 5.30c double arrows.) The double layer of chloroplasts is reduced to a single layer in parts (Figure 5.30c). Some crowding of organelles at the fusion body periphery is observed (Figure 5.30a double arrows.) At the junctions of the contributing cytoplasms, regions of vesiculate cytoplasm can be seen. (Figure 5.30a arrows.)

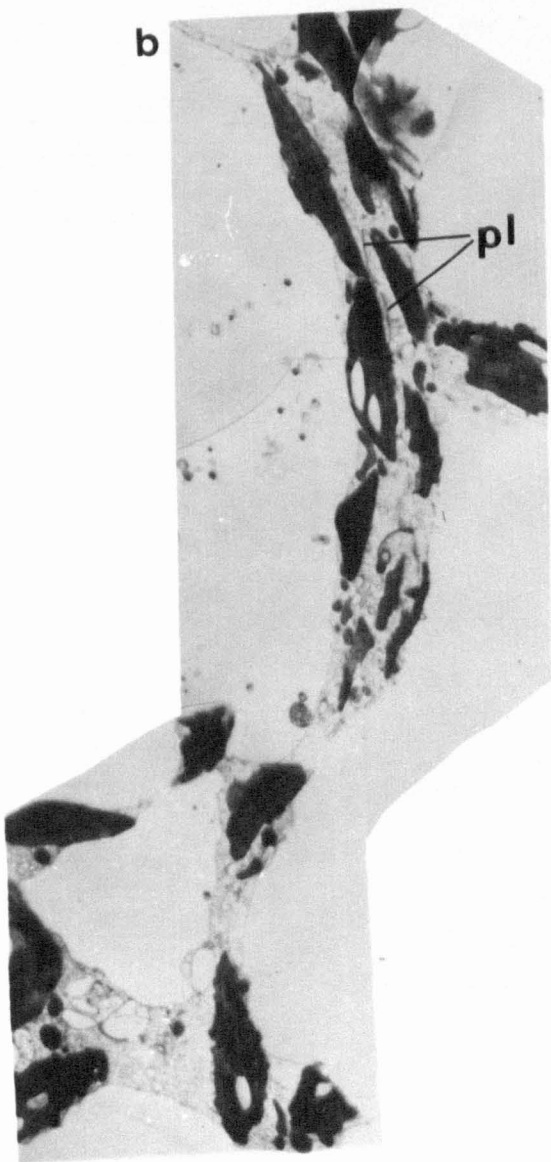
Figure 5.30a x 2,000

Figure 5.30b and c x 9,000

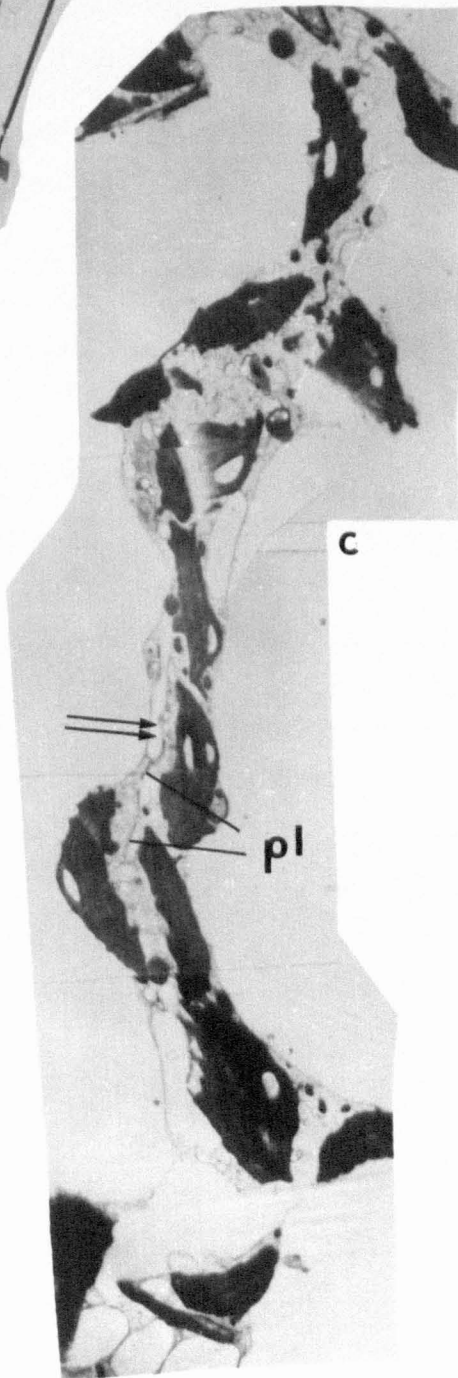
5.30a



b



c



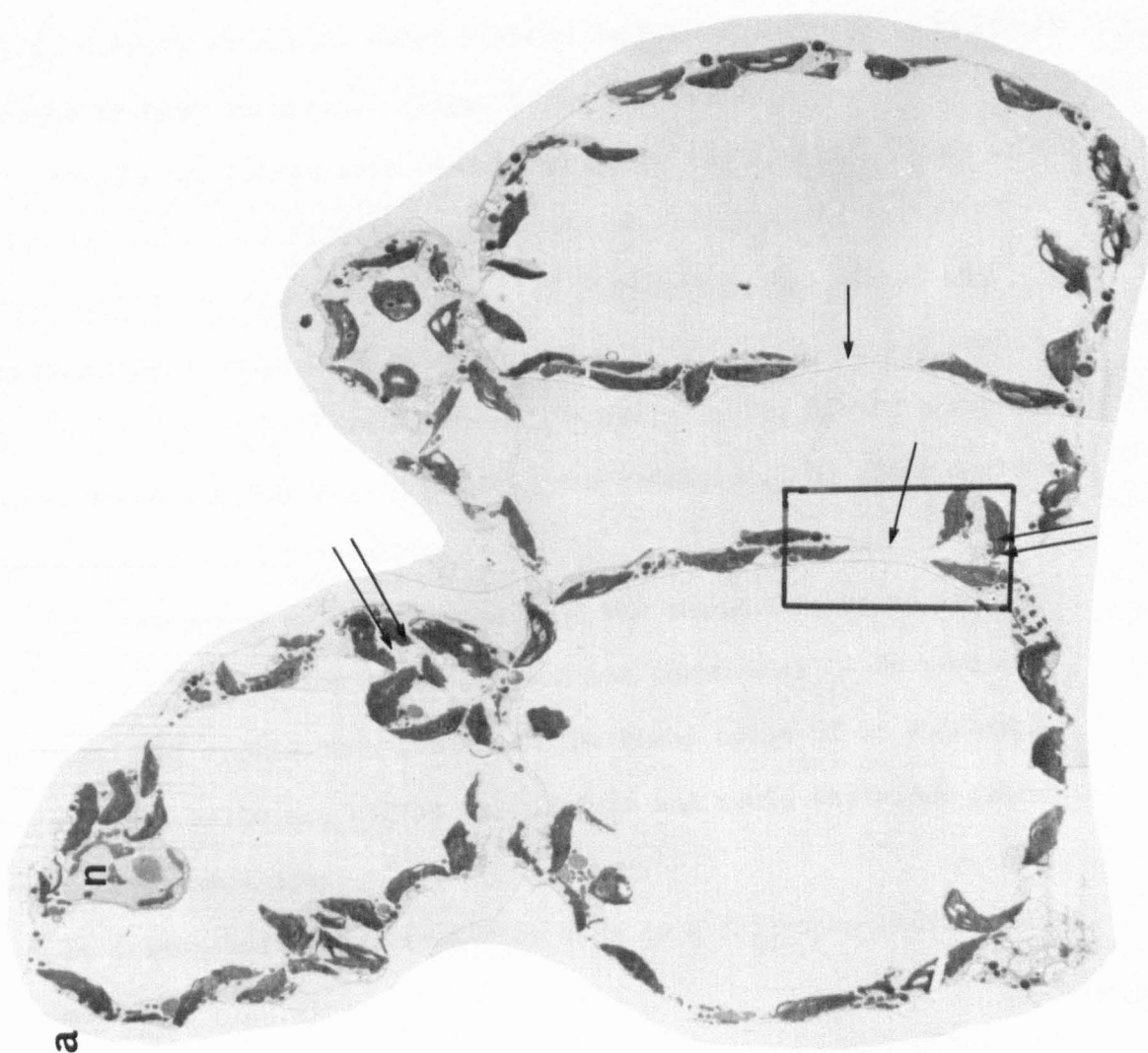
Post fusion events

Figures 5.31a and b A section through a sodium nitrate induced fusion body, showing the fusion interfaces of 4/5 individual protoplasts. Organelle migration away from the fusion regions causes cytoplasmic thinning (Figure 5.31a arrows) with organelle 'crowding' towards the periphery (Figure 5.31a double arrows). Figure 5.31b shows an enlarged region in which the original plasmalemma remains unfused (double arrows).

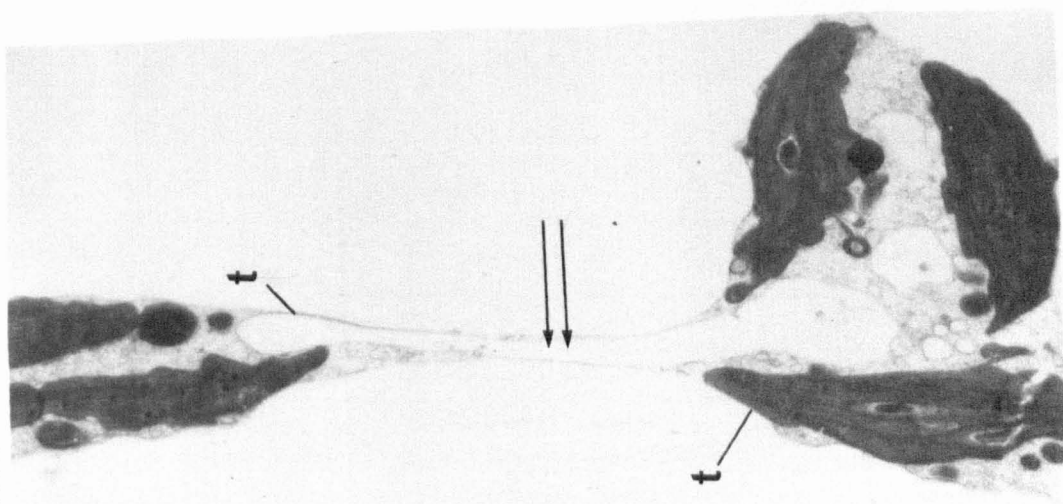
(t = tonoplast)

Figure 5.31a x 2,000

Figure 5.31b x 12,500



5.31b



Many are marked by the original double layer of chloroplasts, (Fig. 5.32a, arrows) whilst in other regions fusion of adjacent vacuoles is thought to have occurred. (Figs. 5.32a and 5.33a *)

Few fusion bodies were seen to progress any further. Some of those which did achieve a more advanced stage of rounding-off (at 48 h) demonstrated nuclear aggregation. In Figure 5.34 two nuclei are separated by a broad band of cytoplasm whilst in Figures 5.35 and 5.36 the nuclei lie very close together. However in the latter examples the nuclei and cytoplasm are in a very poor condition. No clear nuclear interconnexions were observed.

The structure seen in Figure 5.37 was thought to be an induced fusion body but further investigation has shown that it is probably the result of spontaneous fusion in the basal cells of an epidermal hair. These cells are highly cytoplasmic and would therefore probably resist preplasmolysis.

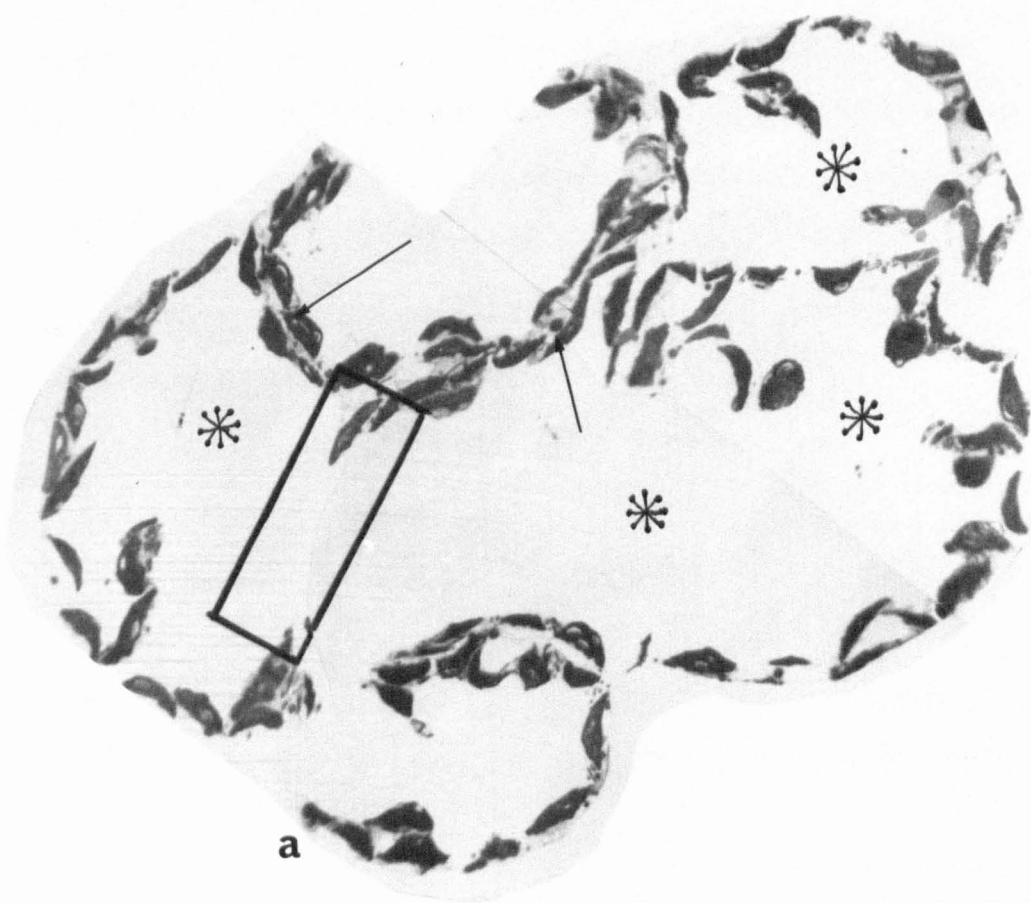
No fusion bodies survived more than 48 h following sodium nitrate treatment.

Post fusion events

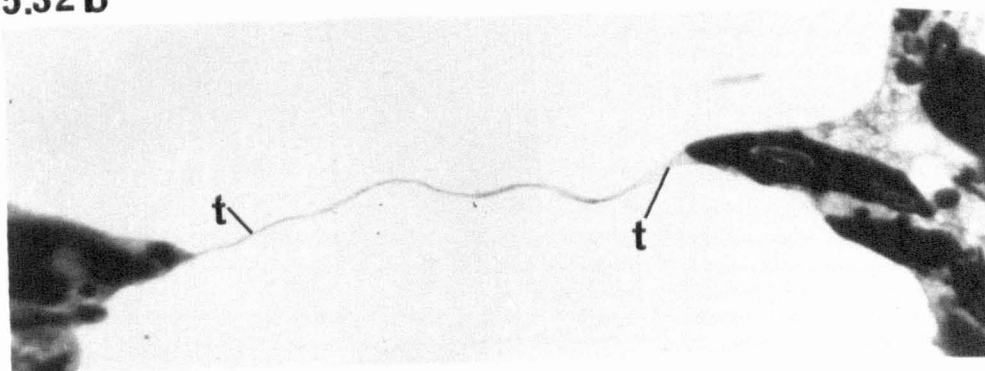
Figures 5.32a and b A region of a large sodium nitrate induced fusion body. The body is in a more advanced stage of fusion than the previous ones. In one region, the vacuoles of the adjacent protoplasts are separated only by the two tonoplasts(t) (Figure 5.32b). Some regions, however, still have the original double layer of chloroplasts, (Figure 5.32a arrows) whilst others appear to have undergone complete(vacuolar)fusion.(Figure 5.32a*)

Figure 5.32a x 2,000

Figure 5.32b x 5,500



5.32 b



Post fusion events

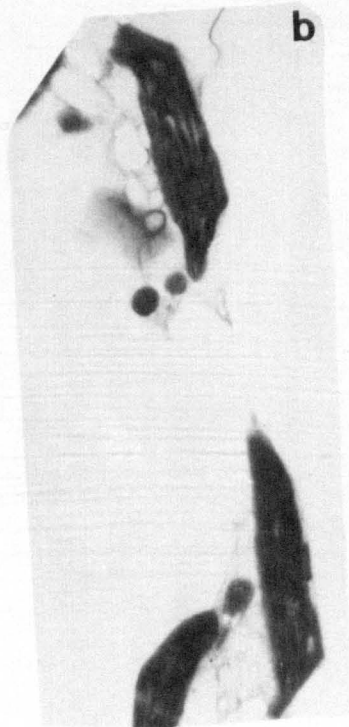
Figures 5.33a and b A region of a large sodium nitrate induced fusion body. As in the previous figure, the changes at the fusion interfaces are not occurring synchronously. Figure 5.33b shows a region where the strand separating the adjacent vacuoles appears to be under stress. In Figure 5.33a, regions where vacuolar fusion has probably occurred are indicated. (*)

Figure 5.33a x 2,000

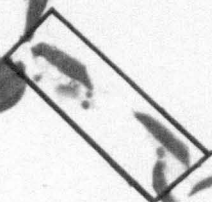
Figure 5.33b x 7,500

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b

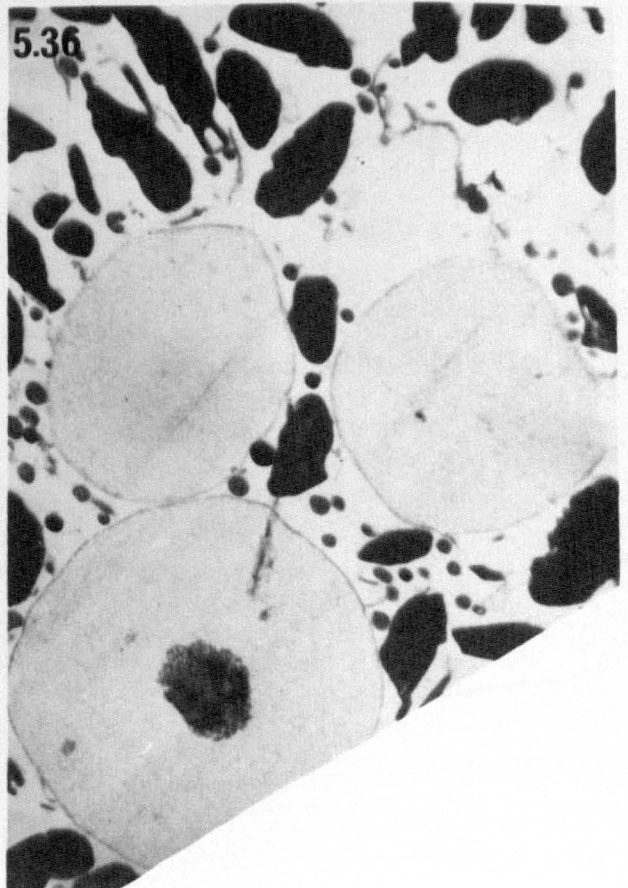
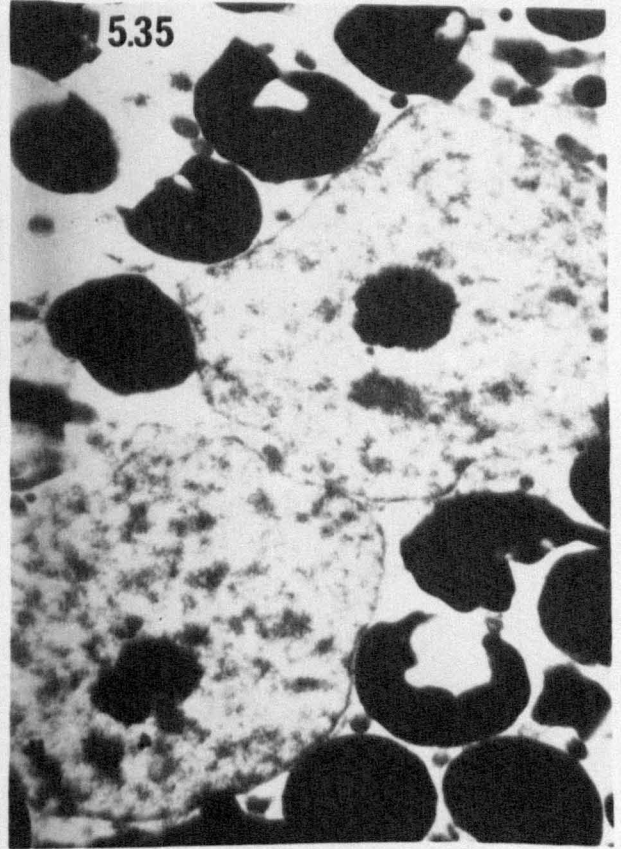
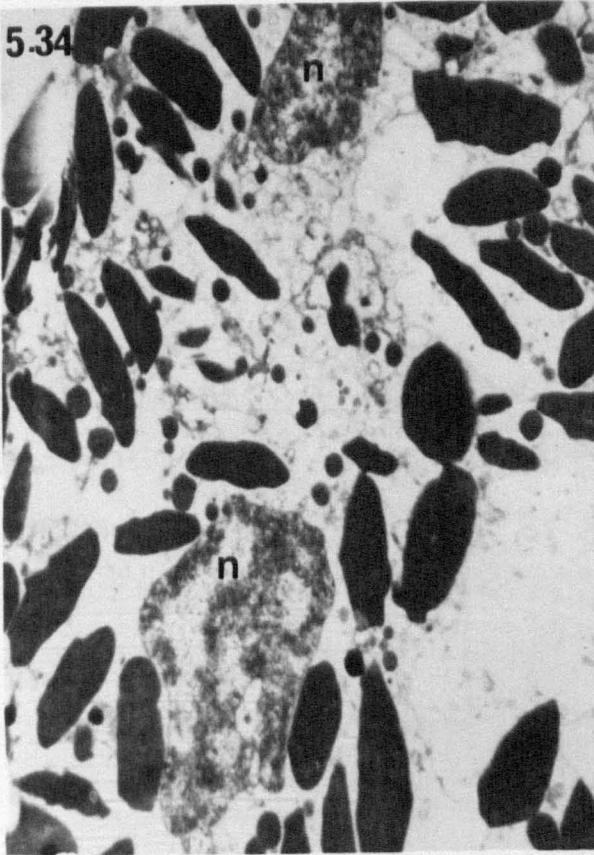


a



Nuclear aggregation in induced fusion bodies

- Figure 5.34 Two nuclei are separated by a region of chloroplast containing cytoplasm. x 6,000
- Figure 5.35 Two nuclei lie very close together. No internuclear connexions are visible. Compare with Figures 3.155 to 3.162. x 8,000
- Figures 5.36 Three degenerating nuclei lie very close together. Again no internuclear connexions can be seen. x 6,000
- Figure 5.37 A multinucleate structure observed in sodium nitrate treated preparations. It is probably a basal hair cell spontaneous fusion body. x 3,000



Summary

(The treatment of protoplasts with inorganic salts)

Protoplasts treated with a variety of inorganic salts were induced to adhere together. A number of effects accompanying and following adhesion were studied in the light and electron microscopes. The fine-structural observation of plasmalemmar 'bubbling' and protrusion formation in preparations treated with certain of the salts probably corresponds to the light microscopic observation of a 'halo' forming around and separating protoplasts.

Other effects observed in the electron microscope include enhanced plasmalemmar staining both generally and locally, the fusion of the plasmalemma with underlying membranes and the formation of vacuolar/tonoplast protrusions. Table 5.1 summarises the individual salt effects.

In sodium nitrate treated protoplasts alone, plasmalemmar adhesion progressed to fusion. Stages in the fusion process and the subsequent fusion body reorganization were observed in the electron microscope. Some nuclear aggregation was observed. Only intraspecific fusion between tobacco(Xanthi) protoplasts was successfully achieved although inter-varietal and interspecific adhesion had occurred.

All salt treatments made the protoplasts susceptible to handling damage. The survival of sodium nitrate induced fusion bodies was poor.

5.2 TREATMENT OF PROTOPLASTS WITH ORGANIC COMPOUNDS. (i)

5.2.1 Urea

Following isolation and washing, tobacco epidermal and mesophyll protoplasts were treated with aqueous solutions of urea. The treatment was carried out similarly to salt treatment. (Section 5.1.1). The urea was used in concentrations ranging from 1 M to 0.4 M. (In all but the most concentrated, the osmoticity was made up with sorbitol.)

The protoplasts were induced to adhere upon compaction. At levels above 0.6 M the urea induced considerable plasmalemmar protrusion formation. In the light microscope there appeared to be a diffuse band around the protoplast. Electron microscopy confirmed this observation and it was shown that the protoplasts rarely adhered by direct membrane contact. Adhesion was transient, protoplast aggregates rapidly dispersing upon flotation in sucrose. (22% w/v). Prolonged treatment had very deleterious effects on the protoplasts.

5.2.2 Lysozyme

Isolated, washed tobacco mesophyll protoplasts were treated with solutions of lysozyme at levels of 10% w/v (100 mg/ml) to 0.1 ppm (1×10^{-4} mg/ml) in 13% w/v aqueous sorbitol. Compaction produced protoplast adhesion at all levels of lysozyme, but aggregates formed in the presence of 0.01% lysozyme or less, rapidly dispersed upon flotation in sucrose. (22% w/v). Those formed during 10 min of treatment with between 0.01% and 10% lysozyme, remained intact after flotation and many survived several washings.

In the light microscope, large faces of adhesion were observed between protoplasts, with possible fusion body formation. Electron

microscopy confirmed the close adhesion but produced no evidence for fusion. Higher levels of lysozyme induced some plasmalemma protrusion formation. (Fig. 5.38a). Little direct membrane contact was observed, densely staining material lying between the plasmalemmae. (Figs. 5.38a and 5.38b, arrows). However when treated with 0.1% lysozyme the amount of the dense material was reduced and direct membrane contact increased. (Fig. 5.38c).

Lysozyme induced aggregates were cultured for up to 4 days but showed no sign of rounding off or other activity.

5.2.3 Concanavalin A.

Following isolation and washing, tobacco epidermal and mesophyll protoplasts were treated with solutions of Concanavalin A (Con A) at levels 1% w/v (10 mg/ml) to 0.15 ppm (1.5×10^{-4} mg/ml) in 13% w/v aqueous sorbitol. The protoplasts were allowed to sink under gravity. Treatment was carried out for periods up to 1 h, after which time the Con A containing solution was replaced by sucrose. (22% w/v). Concentrations of 0.1 to 1% Con A induced protoplast adhesion within 10 minutes (Fig. 5.39) with adhesion being induced within 40 min by concentrations as low as 15 ppm. The majority of aggregates survived flotation and several washings. The size of aggregate increased with Con A concentration, some formed by the most concentrated solutions being too large to handle without disruption. No sign of fusion was observed in the light microscope.

In the electron microscope, very close membrane adhesion was observed. (Fig. 5.40). Occasionally, with the higher levels of Con A, the protoplast membranes were separated by diffuse material. (Fig. 5.41). The plasmalemmar distortion observed with salt treatment was found here also. (Fig. 5.42).

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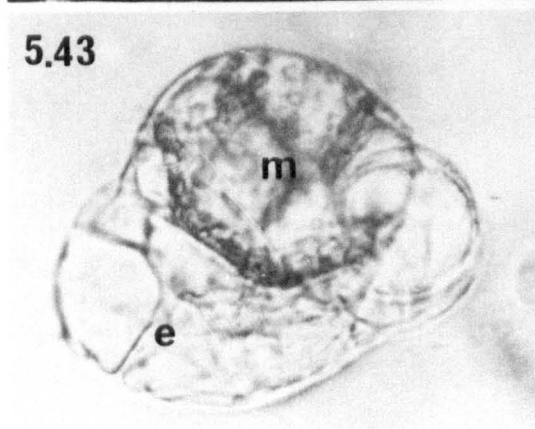
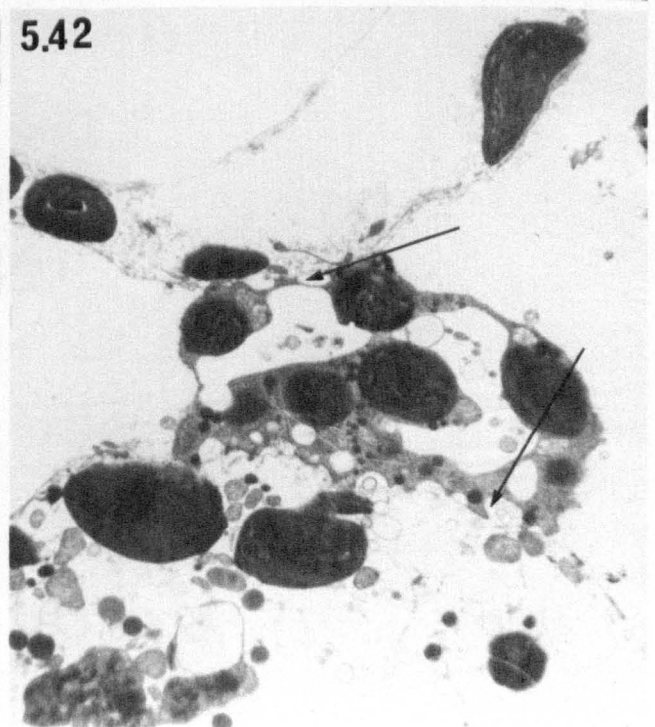
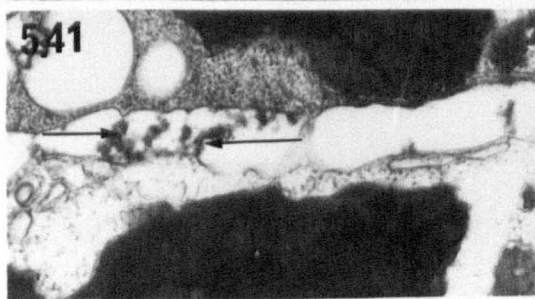
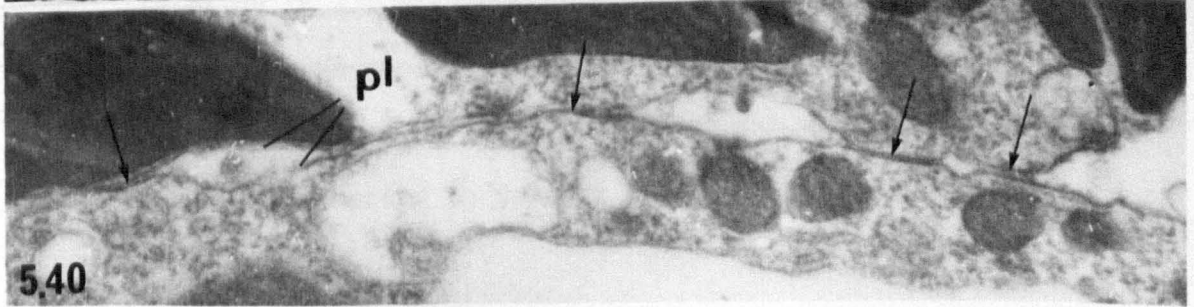
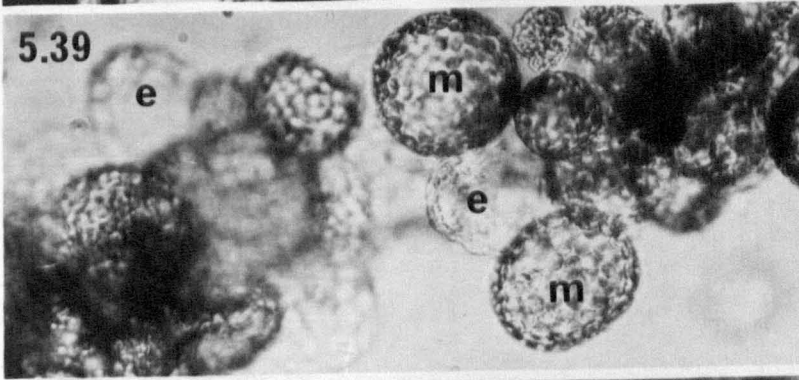
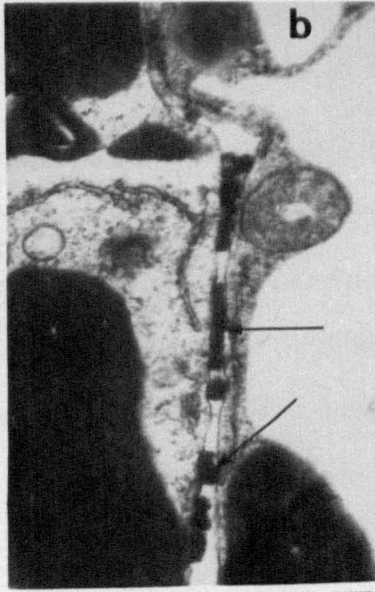
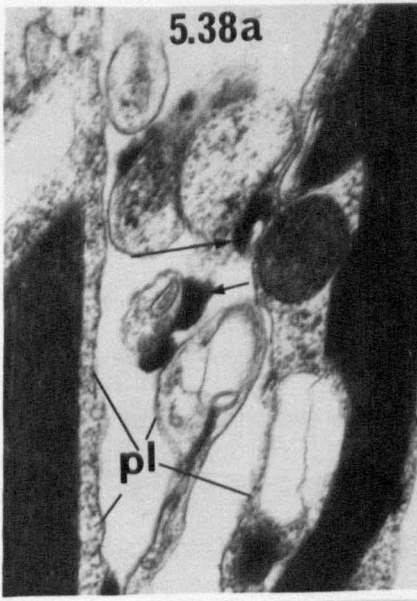
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The treatment of protoplasts with lysozyme and Concanavalin A

- Figure 5.38a Treatment with high levels of lysozyme (see text)
induced plasmalemma(pl) protrusion formation. Dense
material was observed lying on the plasmalemmae(arrows).
x 25,000
- Figure 5.38b Similar to the above, but no protrusions have formed.
A considerable amount of dense material (arrows) lies
between the protoplasts. x 25,000
- Figure 5.38c A reduced level (see text) of lysozyme permits some
direct plasmalemmar adhesion, although the dense
material is still present. x 20,000
- Figure 5.39 A region of a large Con A induced protoplast aggregate.
(Of tobacco epidermal (e) and mesophyll(m) protoplasts.)
x 350
- Figure 5.40 A region of plasmalemma adhesion induced by Con A.
Points of adhesion are arrowed.(pl = plasmalemma)
x 25,000
- Figure 5.41 Two protoplasts are separated by densely staining,
but diffuse material(arrows) which is adhering to
the plasmalemmae. (Con A treated). x 12,000
- Figure 5.42 Three protoplasts adhering after Con A treatment.
The plasmalemma of the central protoplast is
distorted (arrows).This is likely to be a fixation
artifact. x 4,500
- Figure 5.43 A small mixed colony regenerated in solid medium
culture from a Con A induced mixed aggregate of
tobacco epidermal and mesophyll protoplasts.
(e, and m respectively) x 180



Mixed aggregates were cultured for several weeks in a solid medium. The protoplasts remained in contact but visually distinguishable whilst regenerating cell walls and undergoing several divisions. (Fig. 5.43).

Con A, at a concentration of approximately 0.5% w/v in a 22% w/v aqueous sucrose solution was used as the flotation medium for some salt treated protoplast preparations. This treatment stabilized aggregates formed by low level salt treatment. They would normally have been dispersed during the flotation.

5.2.4 IAA and 24-D.

Isolated, washed, tobacco mesophyll protoplasts were treated with these two plant hormones at levels of 1×10^{-3} M to 1×10^{-5} M in 13% w/v aqueous sorbitol. At all levels, some protoplast adhesion was induced but flotation in 22% w/v sucrose dispersed all aggregates. In the electron microscope it was shown that both plasmalemma and tonoplast (as Fig. 5.12) protrusions formed. Protoplast damage was considerable, intact protoplasts frequently adhering to damaged protoplasts and debris.

5.3 THE TREATMENT OF PROTOPLASTS WITH ORGANIC COMPOUNDS, ii

Mixtures of tobacco upper epidermal and mesophyll protoplasts (at a final concentration of 1×10^5 protoplasts per ml) were used in the experiments, which fell into three series:

- a. the treatment of protoplasts with aqueous solutions or suspensions of the compounds,
- b. using emulsions containing, (i) one constituent in the lipid phase,
- c. using emulsions containing, (ii) two to several constituents in the lipid phase.

These also represent something of a chronological progression in the work.

5.3.1 Aqueous preparations

Lysolecithin was used at levels of 0.5% w/v (5 mg/ml) to 50 ppm (0.05 mg/ml) (final concentrations). Initially the lysolecithin was dissolved at double concentrations in either 22% w/v sucrose, 13% w/v sorbitol or 0.6M sodium nitrate. One ml of the solution was added to one ml of protoplast suspension. (the latter in 22% sucrose or 13% sorbitol). The two were mixed by inverting several times.

In sucrose alone as the medium, the protoplasts floated, in the sugar mixture, most gradually sedimented and in the sugar salt mixture and sorbitol alone the majority again sedimented. A rapid adhesion of protoplasts (Fig. 5.44a) was induced by lysolecithin levels above 500 ppm. (0.5 mg/ml). Adhesion did not depend upon compaction although the extent to which epidermal protoplasts sank in the media of higher specific gravity was a good indicator of the force of the adhesion. Adhesion was eventually induced in all preparations.

Within one minute of treatment the higher concentrations of lysolecithin (more than 0.1% (1 mg/ml)) induced a 'skinning' of the protoplasts, exposing the vacuole (Fig. 5.44b) which itself eventually burst. The reaction was most marked in the sedimented and sodium nitrate containing preparations. Again all preparations eventually induced this effect. It was attempted to control the reaction by: (a) using TMS medium in place of a sugar or salt; (b) cooling the preparations (from room temperature to 10°C); (c) flooding with fresh medium; or (d) by the addition of bovine serum albumin (1% w/v; final concentration). However all attempts failed. Lysis appeared to be inevitable upon protoplast adhesion.

The retinol containing preparations should perhaps be included in the following section but since the emulsion/dispersion was prepared somewhat differently it is included here for convenience.

Retinol was initially dissolved at 2% w/v in nitrogen saturated absolute alcohol and then further diluted with a sorbitol solution (13% w/v) to concentrations ranging from 0.1% w/v (1.0 mg/ml) to 50 ppm (0.05 mg/ml). The mixtures were shaken vigorously for 1 min and used immediately. One ml of the suspension was added to one ml of protoplasts suspended in sorbitol (13% w/v). (Thus the retinol concentrations were halved). The protoplasts were dispersed by inverting several times then allowed to settle.

The protoplasts separated cleanly into two fractions, an upper floating layer of epidermal and a sedimented layer of mesophyll protoplasts. No protoplast adhesion was noted. The addition of sodium nitrate (0.3M final concentration) did induce adhesion but this was attributable to the salt alone. The preparations treated with 100 ppm of retinol and above showed some protoplast expansion (Fig. 5.44c) which made them very susceptible to

handling damage and induced bursting after 4h.

5.3.2 Lipid emulsions, i.

Emulsions of glyceryl dioleate, glyceryl trioleate or oleic acid with 13% w/v sorbitol or 0.6 M sodium nitrate were prepared using the methods described in Section 4.4.3. In each case 0.5 ml of the organic compound was added to 100 ml of the aqueous solution. Emulsions prepared manually, or using a vortex mixer, were very unstable, breaking down rapidly, and in so doing creating mobility in the protoplast suspension. It is considered that this largely accounted for the apparent aggregation of protoplasts. (Fig. 5.45a). In this figure some large lipid droplets can be seen (arrows). The aggregates dispersed if disturbed.

The only long term effect of treatment with sugar based preparations appeared to be a loss of spherical outline and with salt based preparations the effects typical of sodium nitrate treatment (Section 5.1) were observed. Ultrasonically prepared emulsions produced a very slight but transient protoplast adhesion.

5.3.3 Lipid emulsions, ii.

Ultrasonically prepared emulsions, in which the non-aqueous phase included glyceryl dioleate or glyceryl trioleate with the addition of lecithin and lysolecithin or glyceryl monooleate were used. Examples of two such preparations are given below:

a.	sorbitol 13% w/v	100 ml
	glyceryl dioleate	0.5 ml
	glyceryl monooleate	0.03g

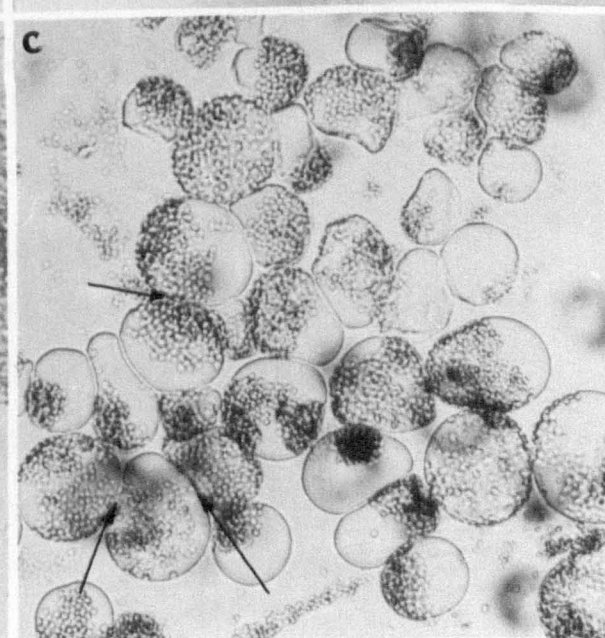
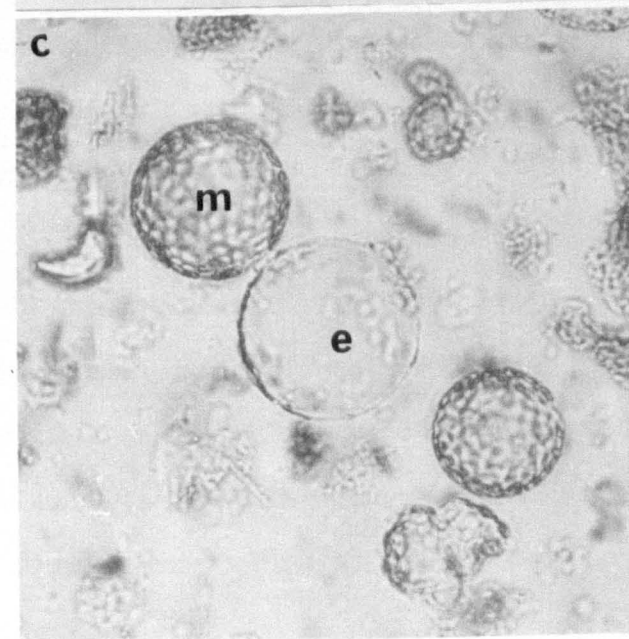
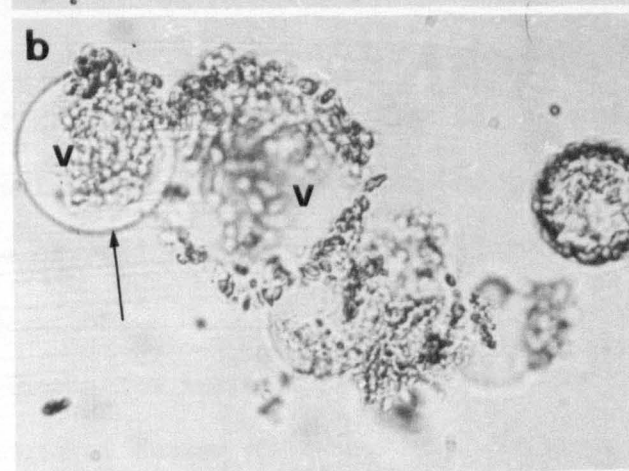
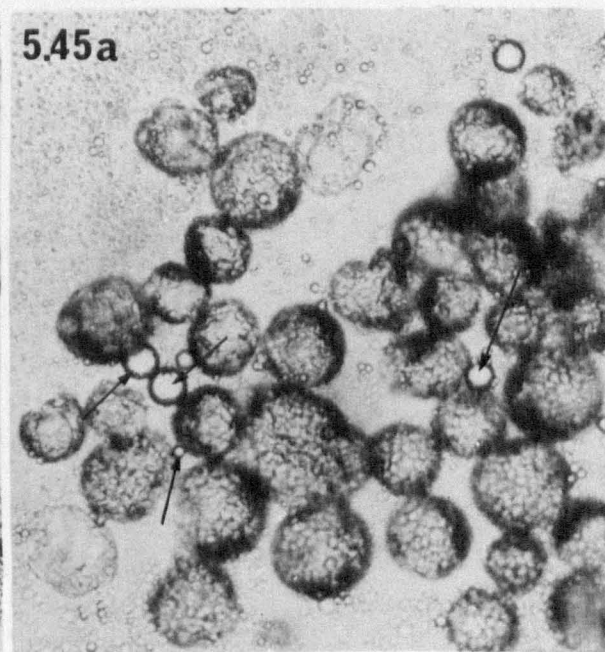
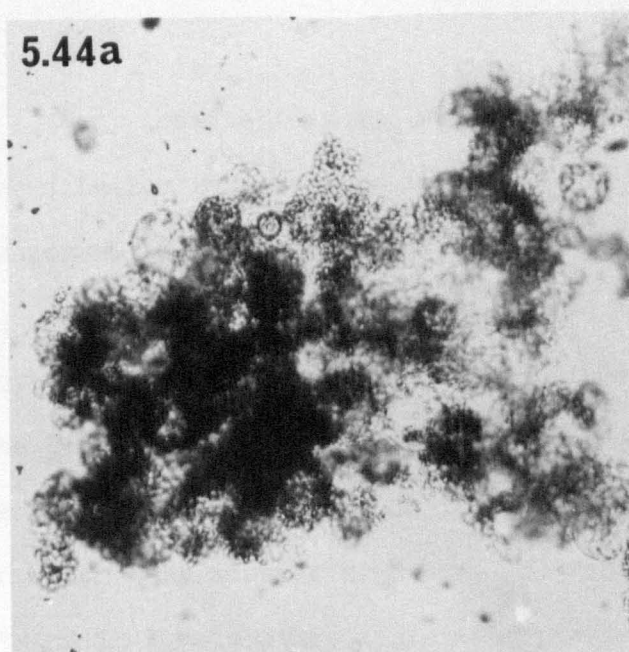
pH approx. 6.

b.	sorbitol 13% w/v	100 ml
	glyceryl trioleate	0.5 ml
	glyceryl monooleate	0.1 g
	lysolecithin	0.05 g
	lecithin	0.03 g

pH approx. 6.

The treatment of protoplasts with certain organic compounds.

- Figure 5.44a A mass of adhering protoplasts, treated with aqueous lysolecithin. Some breakdown is occurring. x 100
- Figure 5.44b Three adhering protoplasts, treated with aqueous lysolecithin. The plasmalemmae and cytoplasm are 'skinning' from the vacuoles(v). The exposed tonoplast of one protoplast is arrowed. x 220
- Figure 5.44c Three protoplasts, two tobacco mesophyll, one epidermal, treated with retinol. The protoplasts are showing signs of expansion. Debris from damaged protoplasts can be seen in the figure. x 200
- Figure 5.45a A loose aggregate of protoplasts treated with a poorly dispersed lipid emulsion(see text for details). Large lipid droplets can be seen(arrows). x 180
- Figure 5.45b Two protoplasts adhering after treatment with an ultrasonically prepared lipid emulsion.(again see text for details.)The protoplast to the left shows some expansion and organelle aggregation. x 250
- Figure 5.45c Preparation as above: a larger mass of protoplasts. The organelle aggregation and possibly 'skinning' is again occurring. Large flattened faces of adhesion between protoplasts are arrowed. Some fusion may have occurred. x 180



Many combinations and varying concentrations were tried. No accurate quantitation was possible in terms of specific effects or protoplast response. General reactions are therefore described.

The protoplasts were concentrated into a very small volume of sorbitol (13% w/v) and some 20 times that volume of emulsion was added. The protoplasts were dispersed in the emulsion and then allowed to sediment. A thick layer of loosely compacted protoplasts frequently formed in which many adhered together. The adhesion appeared to be particularly strong in lysolecithin containing preparations.

An aggregation of the protoplast organelles was commonly observed. (Fig. 5.45b). This was possibly an expansion effect or related to the 'skinning' effect described earlier (Fig. 5.44b). Figure 5.45c shows an extreme example of this. (Produced by a lysolecithin containing emulsion). Many of the protoplasts have lost their spherical form and are adhering by large flattened faces (arrows). Points which could be localised areas of fusion are observed. However, it is considered that in a preparation such as this where the protoplast organization is considerably disrupted, fusion could occur just as a stage in a general breakdown of the protoplasts.

The addition of bovine serum albumin arrested the reaction somewhat but the replacement of the emulsion by TMS medium had a similar effect. Eventual lysis could not be prevented. Increasing the temperature (to 35°C) accelerated the reactions but cooling (to 10°C) did not appear to do more than increase the thresholds of activity of the constituents of the emulsions. Thus no real control over the emulsion effects could be exercised.

All of the above preparations were examined in the electron microscope. A general poor condition was observed, cytoplasmic breakdown being common. No clear examples of fusion in any form were apparent.

5.4 TREATMENT OF PROTOPLASTS WITH SENDAI VIRUS.

5.4.1 General protocol.

Isolated tobacco epidermal and mesophyll protoplasts were washed and then suspended at required concentrations in a sorbitol solution. (14% w/v). Small volumes of Sendai virus suspension, covering a range of concentrations, were prepared by either centrifugally concentrating the stock preparation or diluting it with PBS.

One volume of virus suspension was added to 9 volumes of protoplast suspension and the two were thoroughly mixed by inverting several times. The protoplasts were allowed to sink under gravity or aided by centrifugation. (2 min, 150 x g). After 5 or 10 minutes of virus treatment the virus containing medium was replaced by a sucrose solution (22% w/v) and the protoplasts were allowed to float to the surface.

Sodium nitrate (0.4M) was used (in place of sorbitol) in combination with the virus in some preparations.

5.4.2 Virus effects, (i) commercially prepared virus.

The virus alone failed to have any effect on the protoplasts, detectable either in the light or electron microscope. No difference was observed between the ~~least~~ and most concentrated preparations used. (400 and 4,000 HAU, final titre, respectively.) The presence of virus particles in all preparations was confirmed using the negative staining technique. (Valentine and Horne, 1962). However, when sodium nitrate was included during the virus treatment, an adhesion of virus particles to the protoplast plasmalemma was induced. (Fig. 5.46). A brief treatment (5 min) induced viral adhesion without sustained protoplast

adhesion. The viral adhesion survived several washings.

5.4.3 Virus effects (ii) laboratory prepared virus.

The two laboratory prepared virus cultures were used in a range of concentrations from 50 HAU to 20,000 HAU (final titre). The most concentrated virus had a rapid and marked effect on the protoplasts. Protoplast adhesion was induced within 1 min. (Fig. 5.47a), followed by a loss in spherical outline and finally, protoplast breakdown. (Figs. 5.47b and c).

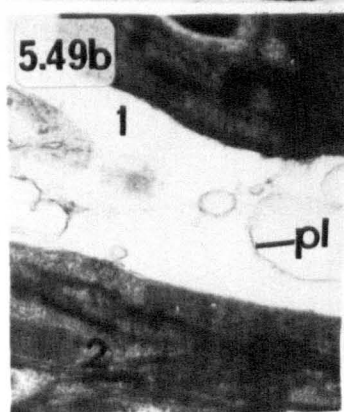
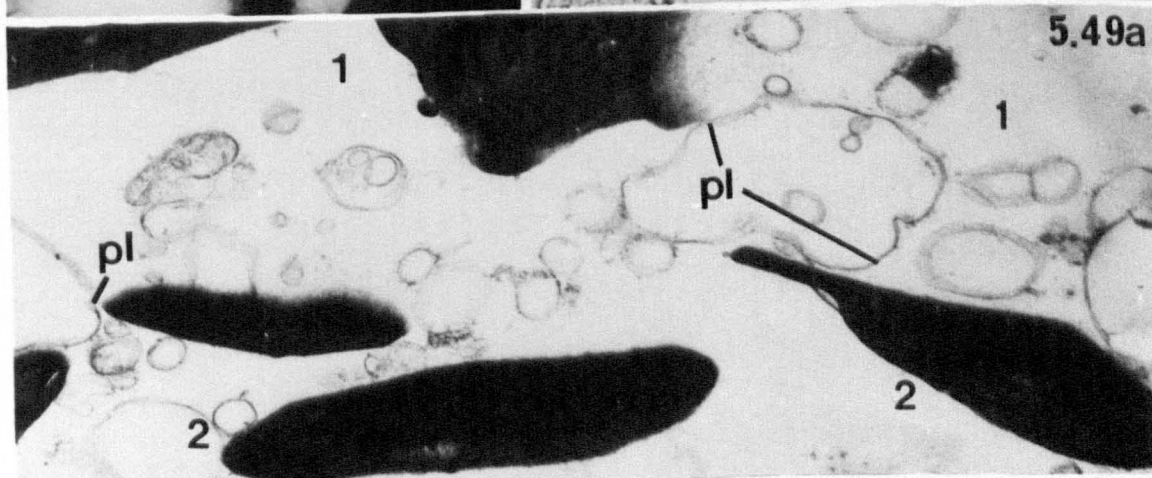
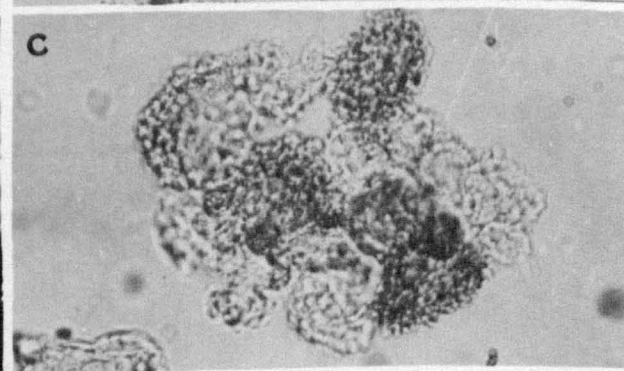
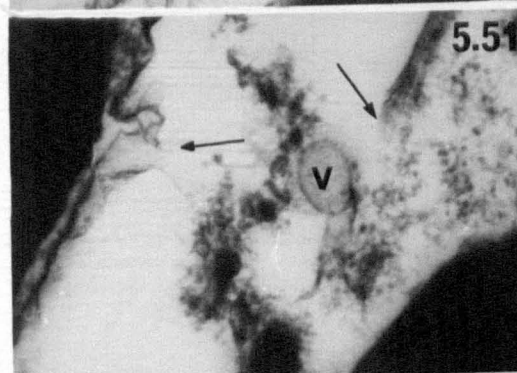
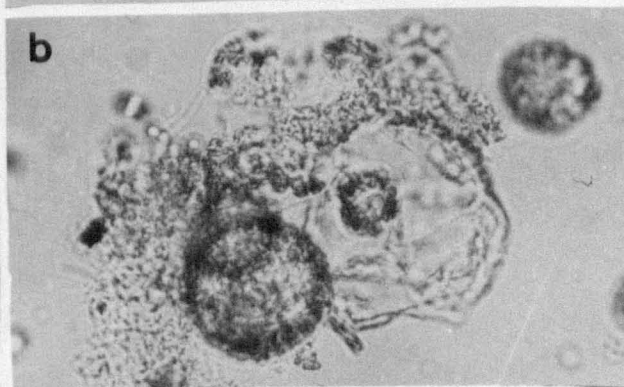
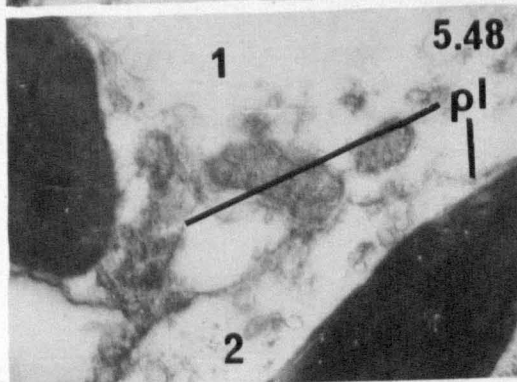
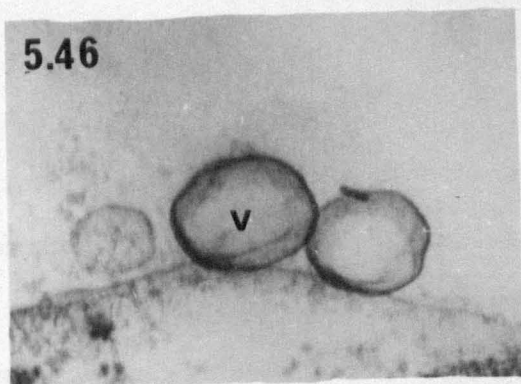
Observation of the process in the electron microscope showed that both viral adhesion and direct plasmalemmar adhesion between protoplasts (Fig. 5.48) were induced initially. This was followed (at approx. 5 min) by the formation of broad membrane connexions between protoplasts. (Figs. 5.49a and b). However at this stage there was invariably the loss of much of the protoplast contents. This condition corresponds closely to the first observations made of sodium nitrated induced protoplast fusion. (Fig. 5.50).

Treatment with virus at lower titres (5,000 HAU and below, final titre) induced some protoplast adhesion but the protoplast aggregates were rapidly dispersed by flotation. However the adhesion of virus particles to the plasmalemma was observed often associated with localised membrane damage. (Fig. 5.51). This adhesion was observed at titres as low as 500 HAU.

In general, the virus prepared at Nottingham appeared to have an activity some double that of the donated virus. Similar effects were observed with the latter, but longer treatment, aided by centrifugation was required. Inactivated virus showed some decline in protoplast

Treatment of protoplasts with Sendai virus

- Figure 5.46 The adhesion of (commercially prepared) virus particles, (v) to a protoplast plasmalemma. x 80,000
- Figure 5.47a Tobacco epidermal and mesophyll protoplasts induced to adhere together by treatment with laboratory prepared virus. (1 min after addition of virus.) x 375
- Figure 5.47b Material from the above preparation, after 8 min of treatment. x 375
- Figure 5.47c Material from the above preparation, after 15 min of treatment. x 375
- Figure 5.48 Direct plasmalemma adhesion between protoplasts treated with laboratory prepared virus. (pl = plasmalemma.) x 22,500
- Figure 5.49a The formation of a broad membrane bridge between virus treated protoplasts. (individual protoplasts are numbered.) (pl = plasmalemma.) x 12,000
- Figure 5.49b As above. x 8,000
- Figure 5.50 An early observation of sodium nitrate induced fusion. The protoplasts are in a similar condition to the above preparation. x 5,500
- Figure 5.51 The presence of adhering laboratory prepared virus (v) near a region of plasmalemma damage. (arrows). x 45,000



response but the general effects were similar. The lower protoplast concentration used (1×10^5 protoplasts per ml.) reacted to lower virus titres than did the more concentrated (5×10^5 protoplasts per ml) preparation although in the former a longer treatment (10 min plus) was often required. In all cases, the virus effect was not greatly increased by the addition of sodium nitrate.

Summary

(Induced protoplast adhesion fusion and lysis)

A number of compounds induced protoplast adhesion in addition to the inorganic salts. Aggregates induced by Concanavalin A treatment survived considerable washing and regenerated to form mixed colonies. Lysozyme had a similar but more reversible effect. However unlike in the above example little direct membrane contact was achieved, dense material lying between the plasmalemmae.

The plant hormones IAA and 24-D induced considerable protrusion formation and transient adhesion.

The use of aqueous preparations of lysolecithin and lipid emulsions containing lysolecithin will induce protoplast adhesion but particularly in the former, lysis followed. Emulsions lacking lysolecithin were less effective in inducing adhesion. An aqueous dispersion of alcoholic Retinol produced bursting of the protoplasts, no adhesion at all was induced.

Commercially prepared Sendai virus failed to induce any protoplast response. In contrast, laboratory prepared virus induced protoplast adhesion membrane fusion and eventual lysis.

Thus protoplast adhesion can be induced using a number of agents, but few treatments lead to fusion and some lead to lysis, indicating that greater control of the reactions is necessary

CHAPTER SIX

DISCUSSION

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6.1 PROTOPLAST ISOLATION

The transformation from cell to isolated protoplast appears, superficially, to be a very simple process, involving plasmolysis and cell wall removal. Upon closer analysis, however, the end product, the isolated protoplast would appear to be rather different from that which existed within the tissue.

6.1.1 The plant cell.

The tissues used in this study fall into two categories; monocotyledonous tissues with relatively undifferentiated^{iat}, actively dividing cells and those with highly differentiated, non-dividing cells - mainly dicotyledonous. The tissue differences are reflected in the degree of vacuolation of the cells, their organelle content and obviously degree of differentiation, and the general cellular interrelationships. (Sections 3.1.1 and 3.3.1).

There are however features common to both tissue types. The existence of the symplasm is confirmed structurally by the existence of plasmodesmata. At a finer level there is a continuity between organelles, (Robertson, 1959) and the e.r. maintains three-dimensional relationships within the cytoplasm assisted by organelle/e.r./plasmodesma connexions. (Stadelmann, 1956). The symplasm exists as a functional entity, continuity being essential to the metabolic processes occurring in the tissue as a whole. (Arisz, 1969).

The plasmalemma, rather than being an inert interfacial membrane is active both synthetically (Villemez, McNab and Albersheim, 1968), and physically, considerable membrane turnover occurring in some tissues. (Mahlberg, 1972). It also plays an important part in regulating fluxes between the cytoplasm and its environment. (Northcote, 1972). The plant

cell wall is essential structurally and is to some extent involved in transport processes. (Lampert, 1970). The existence of a glycocalyx-like intervening layer (Roland and Vian, 1971) adds further to the properties of the protoplast/cell wall interface.

Finally one must consider the extent to which cells are physiologically inter-dependent in a differentiated tissue. Thus we have a picture of a highly complex system in which structural integrity plays a very important part in the maintenance of normal functioning.

6.1.2 The trauma of isolation

Protoplast plasmolysis is brought about by the action of the osmoticum in use, although it commonly occurs in the presence of cell wall degrading enzymes. The transformation from a turgid to a plasmolyzed state produces a dehydration of the cytoplasm, a reduction in vacuolar volume and a reduction in the surface area of the protoplast.

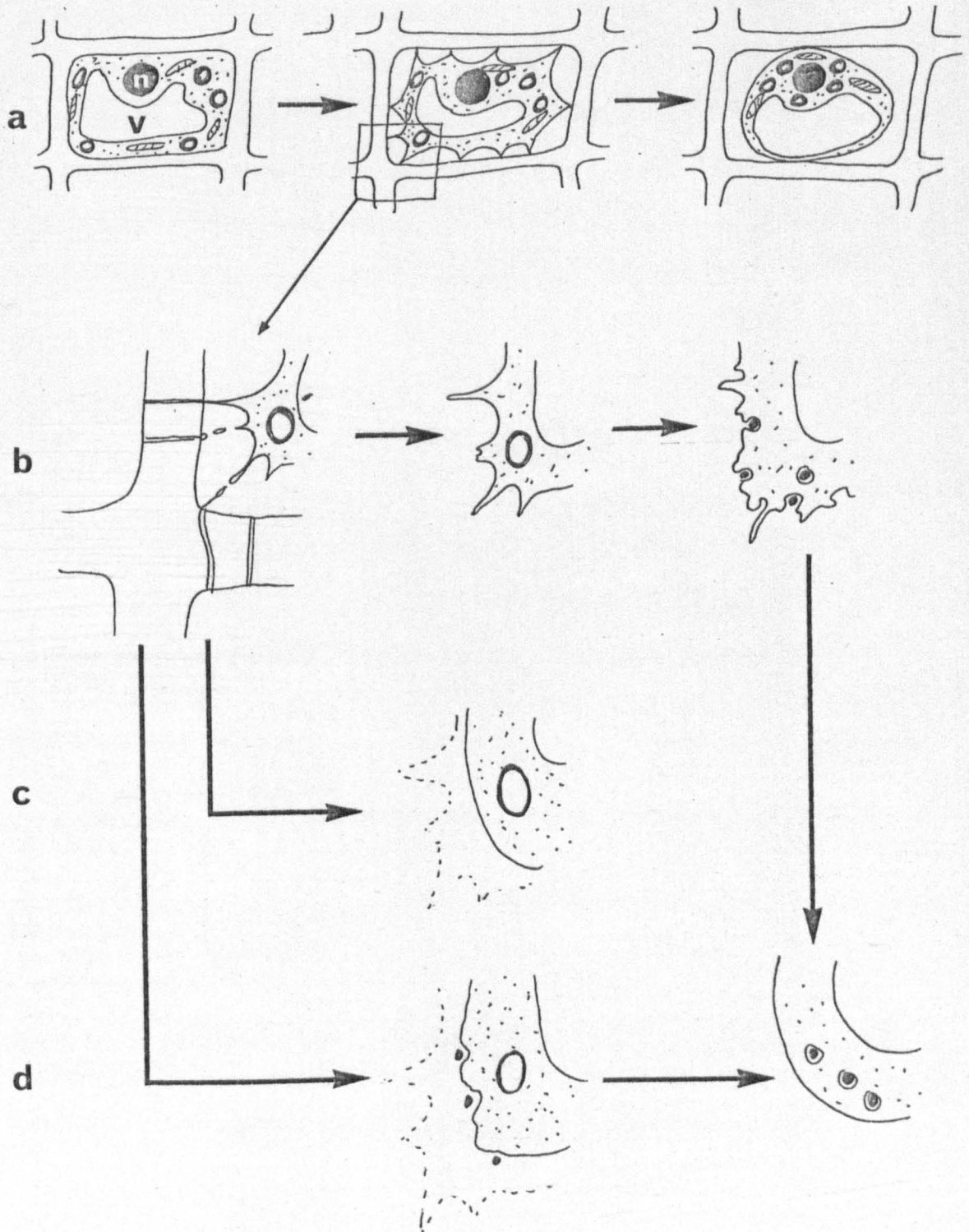
The gross effects of plasmolysis tend to overshadow the fine-structural and physiological effects although the latter may be the more consequential. Research into the effects of water stress have shown that considerable changes can be induced, some at levels to which plants are commonly exposed. (Nir, Klein, and Poljakoff-Mayber, 1969). Condensation of chromatin, and in the extreme nuclear membrane breakdown, the depolymerization of polysomes, cytoplasmic concentration, exhibited as increased ribosomal concentration, changes in chloroplast and mitochondrial form and electron-density, and a reorganization of e.r. are described. Many of these effects have been observed in the present study, (Figs. 3.28, 3.76, 3.86 and 3.133) and are described by Prat (1972) and Gigot, Schmitt and Hirth (1972) as effects of protoplast plasmolysis. These latter workers claim that the changes are reversible.

The separation of the plasmalemma from the cell wall may produce some changes in enzyme activity. Nir et al (1969) suggest that membrane bound enzymes may become separated from cofacting enzymes in the cell wall. The actual reduction in membrane area is considered to impair synthetic enzyme activity. (Grout, 1973).

In regenerated protoplast cultures it is necessary to deplasmolyse to maintain cell wall synthesis and mitosis. (Section 3.4; Frearson, personal communication; Pearce, 1972). However, the two enzyme systems studied by Pilet, Prat and Roland (1972), namely R Nase and transaminase of onion root protoplasts were not affected by plasmolysis alone. (see below).

Considerable surface area to volume ratio changes can occur during plasmolysis. The original shape of the protoplast varies from a simple cube, e.g. in the oat, root-tip cell, to the highly irregular branching tobacco spongy-mesophyll cell. Plasmolysis in the former, to a sphere of diameter equal to the length of side of the cube, involves a surface area reduction of some 48% with an equivalent reduction in volume. However the latter, irregularly shaped cell, plasmolysing to a similar extent would show a proportionately greater reduction in surface area.

Some degree of concave plasmolysis commonly precedes total withdrawal of the plasmalemma from the cell wall. (Fig. 6.1a, Stadelmann, 1956; Pearce, 1972). This must obviously produce an initial stretching of the plasmalemma which can only be relieved by plasmodesmatal breakage. The reduction in tension thus caused would be conducive to plasmalemmar invagination and uptake of material. (Fig. 3.75, Fig. 6.1b; Davey and Cocking, 1972). A scheme in which plasmodesmatal breakage occurs at an early stage in plasmolysis permitting area and volume reduction to occur



- Treatment of the tissue with a hypertonic solution results in the detachment of the plasmalemma from the cell wall. Initially, many plasmodesmata remain intact, thus causing concave plasmolysis. Eventually the plasmodesmata break and the protoplast becomes rounded.
- There is considerable tension in the membrane during concave plasmolysis. Plasmodesmatal breakage causes a sudden reduction in tension. The excess plasmalemma thus created becomes invaginated and material can be taken up.
- If the accommodation of excess plasmalemma occurred immediately after plasmodesmatal breakage, no uptake could occur.
- A latent reduction in surface area would permit some uptake.

simultaneously would not allow uptake on the scale observed, (Fig. 6.1c) unless area reduction were somewhat latent. (Fig. 6.1d).

Sub-protoplast formation, including the attachment of cytoplasmic fragments to broken plasmodesmata, (Figs. 3.17 and 3.58) would accommodate some excess membrane. Protoplast viability and microscopic evidence point to a considerable membrane healing capacity. The numerous lesions formed by plasmodesmatal breakage and the pinching-off of subprotoplasts would make great demands upon such a system.

There is little evidence for serious structural membrane damage resulting from general plasmalemmar detachment. Prat (1972) has observed some retention of polysaccharide fibrils on the plasmalemma with (possibly) associated lipid droplets. The latter may be indicative of membrane damage, (Fig. 3.87), although Nir et al (1969) consider this to be a reversible plasmolysis effect. A coarsening and thickening of the plasmalemma upon plasmolysis reported by Nir et al (1969) has not been observed here or reported elsewhere and it is considered that without very careful control of fixation and staining conditions, such differences would be difficult to substantiate. (Burgess, Watts, Fleming and King, 1972).

The nature of the osmoticum and the wall digesting enzymes are obviously very important considerations. It has been suggested that sucrose may affect protoplast stability (Woodcock, 1973). This is possibly explained by the eventual deplasmolysis induced by long-term sucrose treatment (Stadelmann, 1956), which might overreach the accommodating capacity of the plasmalemma.

Investigations into the effects of fungal enzymes upon host cells have indicated that pectinase, at least, induces water loss. (Hall and Wood, 1970) electrolyte loss, (Mount, Bateman and Grant-Bassham, 1969)

and eventually cell death. The work of Tribe (1955) suggests that plasmolysis protects the protoplast from pectinase effects. However, Tribe's further suggestion that this might be due to a pectinase substrate in the plasmalemma, is difficult to accept. More recently, Pilet et al (1972) reported considerable reductions in enzyme activity between mechanically and enzymatically isolated protoplasts, implicating a deleterious enzyme effect.

The quoted contaminants of crude commercial preparations give some clues to the action of the enzymes. R Nase lipase and phospholipase, contaminants of Meicelase P in particular, must be considered suspect. Ruesink and Thimann (1965) and Ruesink (1973) reported protoplast damage due to R Nase treatment (at high levels). Partial purification of Meicelase P reduces R Nase levels (Coutts, 1973) and is claimed to produce healthier protoplasts. (Woodcock, 1973). However, the more rapid cell wall degradation produced by the purified enzyme may in part account for this. The effect of potassium dextran sulphate (P.D.S.) in increasing protoplast viability (Takebe, Otsuki and Aoki, 1968) is attributed to its polyanionic effect in selectively binding to the R Nase, thereby protecting anionic sites on the plasmalemma. This is compatible with Ruesink's suggestion (1973) that the R Nase is acting as a cationic detergent.

Protease, lipase and phospholipase have yet to be specifically examined.

In addition to a direct membrane effect, enzyme taken up during plasmolysis could lead to protoplast damage. No amount of post-isolation washing could reverse this. Preplasmolysis may therefore play a dual role in protecting the protoplast, by withdrawing the plasmalemma and inducing preplasmolysis before exposure to the enzymes. The inclusion of culture medium salts in the preplasmolyticum may assist in stabilizing the plasmalemma. A final point must be made in this context. The composition of the enzyme incubation medium after protoplast isolation has yet to be analysed. It

is likely to be highly complex, containing wall and cytoplasm breakdown products, and to be of a higher osmotic concentration than when prepared.

6.1.3 The isolated protoplast.

It is possible to define, within limits, the isolated protoplast. Its organelle content will resemble that of the original cell with some slight reduction in smaller organelles due to sub-protoplast formation. The concentration of the cytoplasm may give the impression of a sudden synthesis of organelles. (Gamborg, Kao, Miller, Fowke and Constabel, 1973). However considering the brief time involved and the physiological state of the protoplast, this is unlikely.

The three-dimensional 'reference points' of the cell are lost upon plasmodesmatal breakage, resulting in some degree of systrophy. Thus the structural interrelationships of the organelles may be greatly altered with some impairment of function resulting from plasmolysis. The cytoplasm will almost certainly contain endocytosed particulate material and solutes, some of which may be harmful.

Depending upon the mode of isolation, the plasmalemma may retain limited amounts of polysaccharide material. To what extent the glycocalyx remains is conjectural. 'Scars' in the form of localised variations in membrane structure or coating, results of healed plasmalemmar breaks, may be evident. (Mayo and Cocking, 1969). Some sub-protoplasts or drawn-out strands of cytoplasm may remain on the protoplast periphery. Generally, the protoplast will bear a net negative charge (Grout, 1973), the charge possibly modified by the binding of foreign ions to the plasmalemma.

The buoyant properties of the near-spherical protoplast will depend upon its origin and the osmoticum, but generally use of these properties will facilitate protoplast manipulation. The latter consideration and some

earlier ones will vary in the case of spontaneous fusion bodies. These points are discussed in some detail later.

The physiological condition of the protoplast is difficult to predict but experience shows that it may require considerable nutrient supplementation. Protoplasts isolated from crown-gall callus (Parthenocissus tricuspidata) for example, require the addition of growth regulators, (IAA and kinetin) in early stages of culture, whereas the parent callus does not. (Scowcroft, Davey and Power, 1973).

The description above would appear rather pessimistic but it explains the failure of some early attempts to isolate, manipulate and culture protoplasts, and underlines the sophistication of current techniques.

6.2 PROTOPLAST INTERACTIONS

The levels of interaction between protoplasts in the whole plant, are limited to those mediated by symplasmic contacts and gametic interactions, (Austin, 1968) between compatible varieties or species. Upon isolation the protoplast is theoretically capable of undergoing a far wider range of interactions. However, it becomes apparent that, although the protoplast is highly manipulable there is a close relationship between its scope of interactions and the natural interactions described above.

6.2.1 Spontaneous fusion: a mechanism

Many workers have not readily recognised the differences between spontaneous and induced fusion, resulting in some ambiguity. A number of examples of the former have been, it is considered, misinterpreted as induced fusion, (Eriksson, 1970; Power, 1971; Fodil, Esnault and Trapy, 1971), with a resulting confusion over the conditions necessary for fusion. Clearly, there is a need to understand fully the nature of the processes involved in spontaneous and induced fusion, to be able to distinguish between the two.

A number of mechanisms for spontaneous fusion body formation have been proposed. The possibility of spontaneous fusion being an expression of multinucleation in the parent tissue, the latter resulting from a high rate of mitosis or nuclear division without cytokinesis, has been a common suggestion. (e.g. Nagata and Takebe, 1971). Nuclear division after protoplast isolation, again without subsequent cytokinesis has been proposed by Reinert and Hellmann (1971). These two suggestions imply that 'fusion' is a misnomer.

Takebe, Otsuki, Honda, Nishio and Matsui (1973), and Schenk and

Hildebrandt (1970) consider that the fusion of single isolated protoplasts, induced by the conditions existing during isolation, could explain the observations of spontaneous fusion. This process would, presumably, be analogous to the 'spontaneous hybridization' of animal cells. (Harris, 1970). A phenomenon again occurring at the time of isolation, but involving some reorganization of the symplasm, is the final suggestion. (Power and Cocking, 1971).

A cursory evaluation of the evidence for and against each of the suggestions is given, followed by an examination of the implications drawn from the results of the present study.

A comparison between the levels of multinucleation in the parent tissue and in the isolated protoplasts, clearly shows that some increase is occurring during protoplast isolation. (Woodcock, 1973). The work of Motoyoshi (1971) using maize endosperm callus, has shown that some binucleate protoplasts could originate from binucleate parent cells but larger multinucleates could not. It is conceded that this mechanism could lead to a low level of multinucleation but no more, and can not explain the facility with which the level can be manipulated. (Section 3.5; Power, 1971; Woodcock, 1973).

A repeated nuclear division without cytokinesis, clearly explains the occurrence of multinucleates in the Daucus carota protoplast cultures examined by Reinert and Hellmann (1971 and 1973). A similar mechanism is considered to explain the observation of other workers (Eriksson and Jonasson 1969; Scowcroft et al, 1973). However the divisions are occurring over a considerable length of time and could not possibly account for the rapid appearance of multinucleates during the isolation of spontaneous fusion bodies.

To consider the third suggestion that fusion of originally separate protoplasts is occurring at some stage during isolation: Some observations of enzyme treated oat root tips, (Figs. 3.16 to 3.18) would suggest that a general membrane breakdown in damaged regions might give the impression of fusion, the protoplasts having been at some stage mutually isolated. Protoplast contact is an obvious prerequisite for fusion. In tissues other than the highly cytoplasmic, such as the oat root, it is considered that any great amount of plasmalemmar contact during protoplast isolation would be unlikely. It becomes more feasible after isolation, when the protoplasts are freed into the incubation medium. The observation by Woodcock (1973) that isolation in the presence of sorbitol rather than sucrose produces a higher level of spontaneous fusion, should be noted. It could be implied that fusion was facilitated by the increased protoplast contact produced by compaction in sorbitol. However, electrophoretic evidence would indicate that even sedimented protoplasts should remain sufficiently separated by charge repulsion forces to prevent fusion. (Grout 1973).

If fusion were to take place in the enzyme incubation medium, fusion could theoretically occur between protoplasts isolated from tissues of two species in the same enzyme incubation medium. Also, there should be no reduction of spontaneous fusion when maceration precedes cell wall digestion in a sequential protoplast isolation, (Coutts, 1973) nor should pretreatments reduce the level of fusion. (Section 3.5.3). However, no interspecific fusion is observed under the conditions mentioned above. (Power, personal communication), and the treatments described do reduce the level of multi-nucleation. (Woodcock, 1973).

One must of course add the corollary that fusion under such conditions must be defined as induced fusion. The constituents of the enzyme incubation medium are as validly described as inducing agents as

those intentionally employed. (Section 4.4).

Accepting that all of the mechanisms described above might be occurring together and optimally, they do not satisfactorily account for the observations described in this thesis, or by other workers. (Prat, 1972; Kao, Gamborg, Michayluk, Keller and Miller, 1973). We are therefore left with the final suggestion that spontaneous fusion is a phenomenon involving a rearrangement of the symplasm, at the time of protoplast isolation. The results of sequential protoplast isolation and preplasmolysis, mentioned above, would support this suggestion since both treatments ensure some degree of fragmentation of the symplasm.

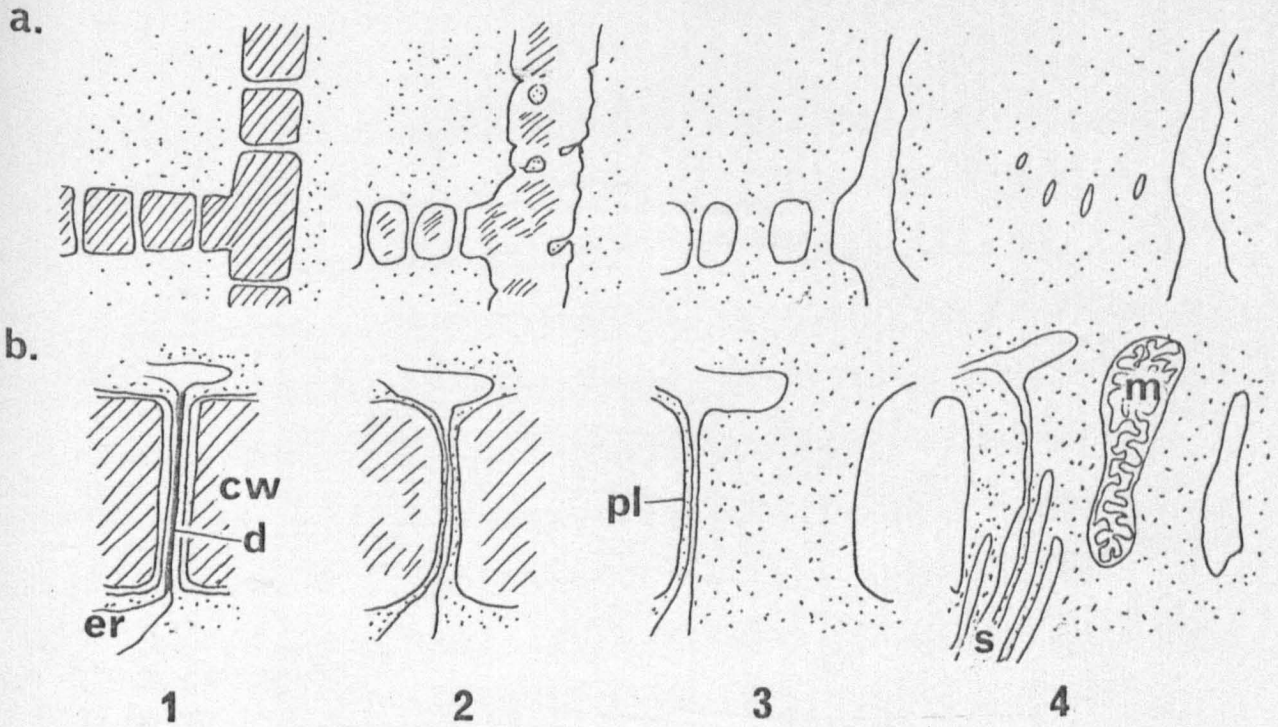
Tissues which produce a high level of spontaneous fusion are observed at early stages in the enzyme treatment to resist plasmolytic breakage of plasmodesmata. (Sections 3.1, 3.3; Prat, 1972). Thus there is a means of retaining the symplasmic continuity despite destruction of the tissue organization, an apparent paradox. The ability of plasmodesmata to form inter-protoplast connexions which could enable organellar transport to take place, depends upon their structure. Some models for plasmodesmatal ultrastructure lack the necessary flexibility, (Robards, 1968; Appendix 1, Fig. A1.1b), whilst others could readily transform under suitable conditions into functional bridges. (Robards, 1971; Appendix 1, Figs. A1.1a, c and d). The latter models contain an element of cytoplasmic continuity, via e.r./plasmodesmatal connexions, which could explain the facility with which organelle transfer takes place. (See also Section 6.3.3).

In fact, protoplast interconnexions which can be positively identified as being of plasmodesmatal origin are observed in the enzyme treated oat root tip, (Section 3.1.3) and in the onion root tip. (Prat, 1972). These interconnexions retain the appearance of expanded plasmodesmata up to a

very advanced stage of protoplast coalescence. In the tobacco leaf, identification of the connexions is not as readily established, but as in the oat root tip their distribution does resemble that of the plasmodesmata prior to enzyme treatment. Thus there is considerable evidence in support of a mechanism for spontaneous fusion, which involves an expansion of plasmodesmata. A schematic sequence of events is presented in Figure 6.2.

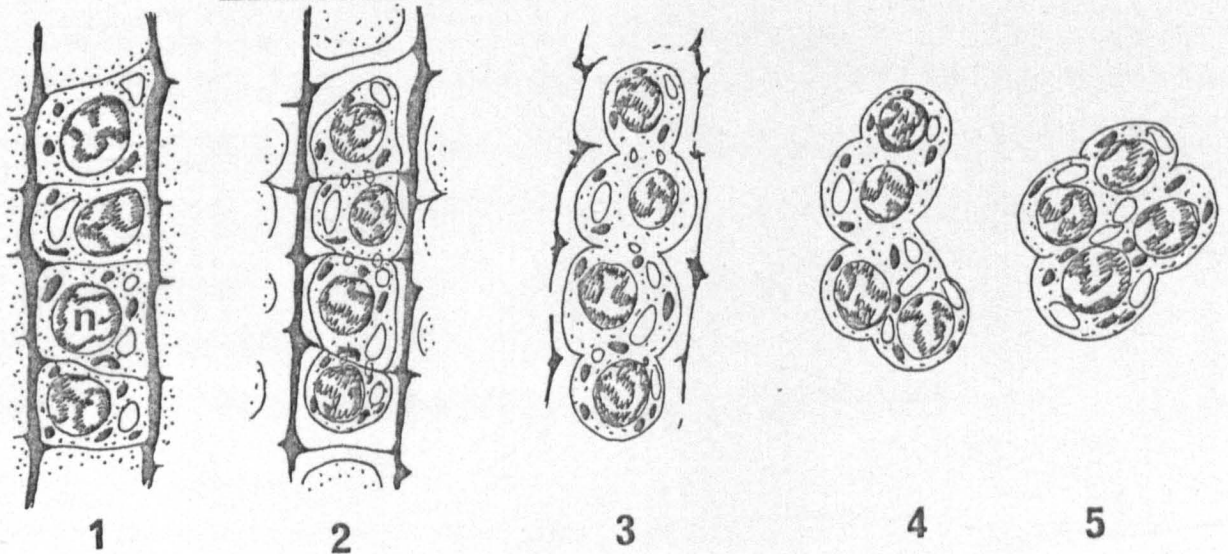
Despite many observations of spontaneous fusion, only one other worker (Prat, 1972) has pointed out the striking relationship between the shape of newly isolated spontaneous fusion bodies and the parent tissue organization. This alone is strong evidence for the involvement of the symplasm in spontaneous fusion. It is fortunate that the tissues investigated in this study showed such marked differences in organization and secondly that the levels of spontaneous fusion were such that the differences could be expressed.

The strong element of linearity in fusion bodies isolated from monocotyledonous tissues closely relates to the plasmodesmatal distribution, (Table 3.1.1), wall thicknesses and pattern of plasmolysis in the parent tissue. The regional variations in the level of spontaneous fusion in the oat root tip (Fig. 3.33) can be attributed to an interaction of these factors. Thus a high plasmodesmatal frequency does not ensure fusion body formation where plasmolysis effects are likely to be extreme, e.g. as in the epidermis of the oat root. The other monocotyledonous tissues studied, provide supporting evidence for a general pattern of fusion (with the exception of the mature maize leaf, Appendix 2.1). A suggested sequence of events occurring during the enzyme digestion of the monocotyledonous tissue is presented in Figure 6.3



- Stage 1. The untreated tissue. Adjacent protoplasts are joined by plasmodesmata.
(c.w. cell wall, d. desmotubule, e.r. endoplasmic reticulum)
- Stage 2. Wall digestion is occurring; some plasmodesmata break, others remain intact and begin to expand. The desmotubule becomes clearer.
- Stage 3. The plasmodesmata expand further, forming broad inter-protoplast bridges. The desmotubule becomes displaced. (pl. plasmalemma)
- Stage 4. The cytoplasmic continuity thus formed permits organelle exchange.
(m. mitochondrion, s. stacked e.r.)

Figure 6.3 Enzyme digestion and spontaneous fusion body formation in monocotyledonous tissues



- Stage 1. The untreated tissue.
- Stage 2. Plasmolysis breaks many longitudinal wall plasmodesmata but many of the plasmodesmata traversing transverse walls remain intact.
- Stage 3. Wall digestion permits the expansion of the transverse plasmodesmata, thus forming a linear (longitudinal) spontaneous fusion body.
- Stages 4 and 5. The fusion body is released from the digested tissues. It rounds off and loses its linear shape.

The tobacco leaf provides an interesting contrast. The structure of the leaf has been described. (Section 3.3.1). The early evolution of small, irregularly shaped fusion bodies, followed by the production of large flat bodies, suggests a progressive digestion of the spongy then palisade mesophyll, the latter being exposed to the digestive enzymes as a unit. Theoretically if complete plasmodesmatal continuity could be maintained a single fusion body could be formed from the entire palisade layer. In fact, the regions of damage in large fusion bodies certainly suggest that fusion body size exceeds the limits of practical handling.

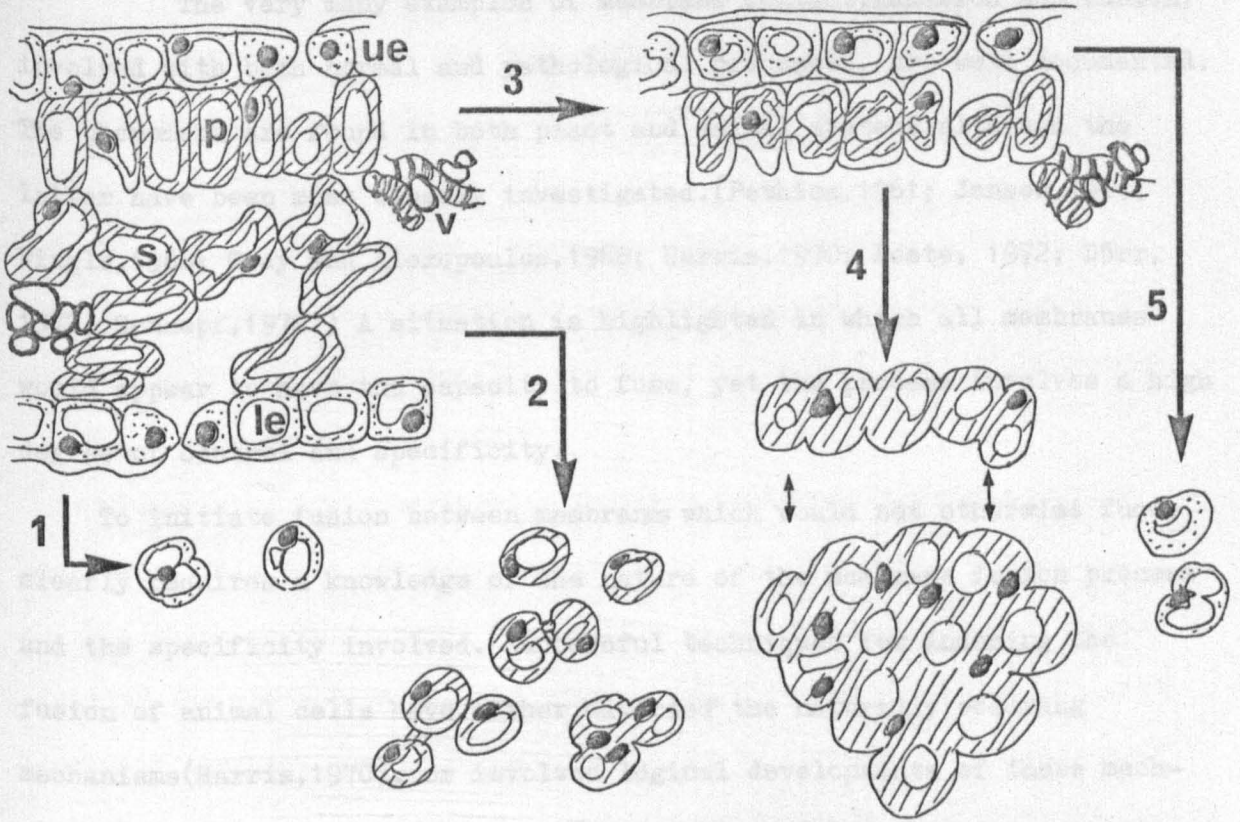
The rather open structure of the spongy mesophyll leads to the frequent liberation of protoplasts connected by unexpanded plasmodesmata. A similar observation has been made by Gamborg et al (1973) of interconnected Ammi visnaga protoplasts.

It is considered that such structures would be very vulnerable and therefore potential fusion bodies could be lost in routine handling. This could explain the observation that isolates produced by partially purified enzyme do not contain significantly higher numbers of multinucleates than those produced by crude enzymes. (Table 3.4). The purified enzyme accelerates wall digestion thereby reducing the risk of plasmolysis damage, but could not accelerate the cytoplasmic reorganizations necessary to the achievement of fusion body stability. In contrast, enzyme treatment at elevated temperatures could accelerate the reorganization as well as the wall digestion. Table 3.5 demonstrates that in some cases this appears to be so. (also, Miller, Gamborg, Keller and Kao, 1971).

The pattern of tissue digestion and protoplast release in the tobacco leaf is schematized in Figure 6.4.

It is interesting to compare the release of spontaneous fusion bodies from higher plant tissues with the release of multinucleates protoplasts from fungal hyphae. (Fig. 6.5).

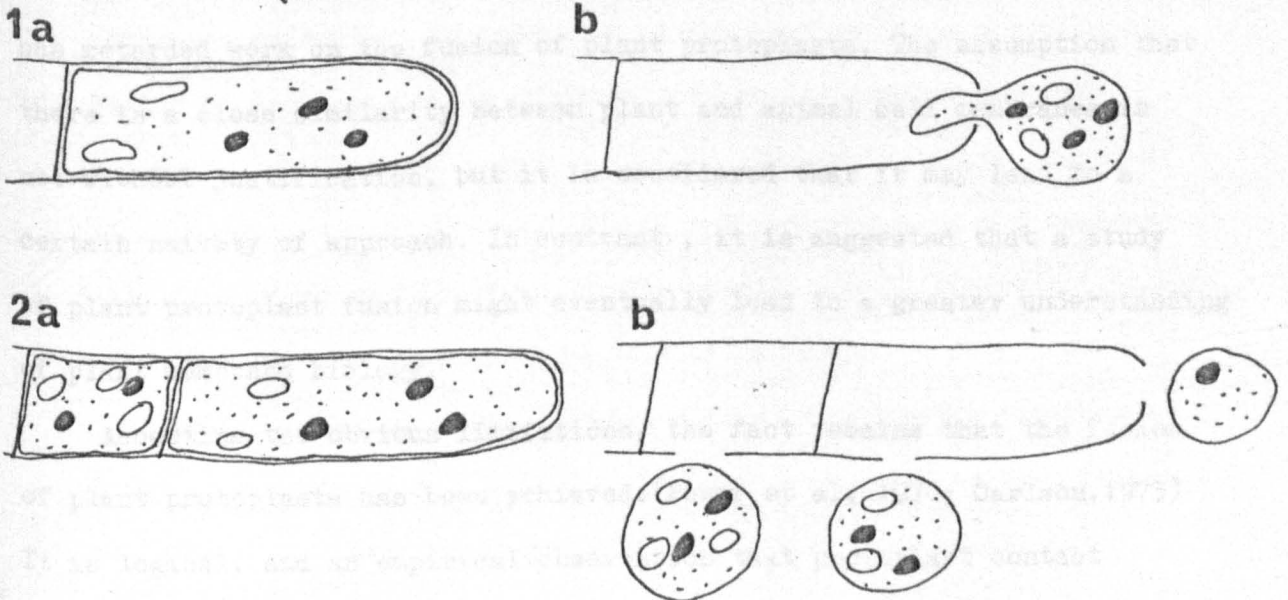
Figure 6.4 The pattern of enzyme digestion and the release of protoplasts and spontaneous fusion bodies: tobacco leaf system.



1. Lower epidermis (le) removed, single protoplasts released.
2. Small spontaneous fusion bodies released from spongy mesophyll (s)
3. Upper epidermis (ue) palisade mesophyll (p) and vascular tissue (v) exposed.
4. Palisade mesophyll releases large flat spontaneous fusion bodies.
5. Upper epidermis releases single protoplasts.

Figure 6.5 Release of protoplasts from fungal hyphal cells

1. from terminal cell,
 2. from terminal and sub-terminal cells.
- (from J.F. Peberdy, Sci. Prog. (1972) 60, 73-86)



a. multinucleate cells.

b. multinucleate and uninucleate protoplasts and sub-protoplasts released.

6.2.2 Induced protoplast adhesion, fusion and lysis.

The very many examples of membrane contact, adhesion and fusion, involved with both normal and pathological processes, are well documented. The phenomena are found in both plant and animal systems although the latter have been most closely investigated. (Pethica, 1961; Jensen, 1964; Dingle, 1968; Gray and Alexopoulos, 1968; Harris, 1970; Poste, 1972; Dörr, 1972; Schnepf, 1972.) A situation is highlighted in which all membranes would appear to have the capacity to fuse, yet the process involves a high degree of control and specificity.

To initiate fusion between membranes which would not otherwise fuse, clearly requires a knowledge of the nature of the membrane fusion process and the specificity involved. Successful techniques for inducing the fusion of animal cells have either 'diverted' the naturally occurring mechanisms (Harris, 1970), or involved logical developments of these mechanisms. (Ahkong, Cramp, Fisher, Howell and Lucy, 1972.) To be more specific, the former uses the fusion inducing capacity of viruses such as Sendai virus, and the latter uses organic compounds with known membrane activity, (including lysis, fusion and endocytosis.)

A considerable amount of knowledge has accumulated on the structure and composition of animal cell membranes. Such a level of understanding has yet to be achieved in the case of plant cell membranes. This in part has retarded work on the fusion of plant protoplasts. The assumption that there is a close similarity between plant and animal cell membranes is not without justification, but it is considered that it may lead to a certain naivety of approach. In contrast, it is suggested that a study of plant protoplast fusion might eventually lead to a greater understanding of plant membrane biology.

Accepting the obvious limitations, the fact remains that the fusion of plant protoplasts has been achieved. (Power et al, 1970; Carlson, 1973) It is logical, and an empirical observation that protoplast contact

and adhesion, whilst being prerequisites for fusion, do not necessarily lead to fusion. An explanation for this, based on the consideration of gross physical factors has been suggested by Power(1971), but the results presented here and by other workers would suggest that rather more complex factors are involved.

Protoplast adhesion and fusion have been induced by a variety of agents, some treatments eventually leading to lysis. Without discussing mechanisms for fusion in detail, the range of induced protoplast interactions reported are reviewed:

Protoplasts are occasionally observed to adhere without specific treatment. This does not necessarily imply that the negative surface charges which normally repel protoplasts have become modified. (Theoretically, the attraction of like charges can occur at a certain membrane proximity. (Pethica, 1961)) It is interesting to compare the pentalaminar formation observed in Figure 5.1 with stages of bacterioid membrane fusion (Gunning, 197), Sendai virus induced fusion of animal cells (Toister and Loyter, 1973) and granule fusion during mast cell secretion. (Lagunoff, 197) Additionally, plasmalemmar fusion must occur during plasmolytic uptake and other endocytotic processes (Grout, 1973) which occur in protoplasts. These points illustrate the fact that the potential for fusion is present in the untreated plasmalemma.

Enhanced protoplast adhesion can be achieved by various treatments. The action of inorganic salts involves the facilitation of membrane contact by the reduction of negative surface charges. Excessive treatment causes a charge reversal, (Grout, 1973). This is borne out by the observed concentration effects (Table 5.1). Little specificity is apparent in terms of the cation used or whether it is uni- or bivalent.

Protoplast adhesion induced by Con A treatment clearly involves a different process (Section 5.2.3) A slight charge effect has been reported (Grout, 1973), but the size of the molecule implies that if attractive

forces were involved, then the molecule would induce adhesion by bridging between the protoplasts, possibly providing a substantial barrier to fusion. At the pH used, the Con A molecule will be particularly large. Thus if it has any potential for inducing fusion at all, it is clear that low concentrations must be used to avoid large deposits lying between the protoplast plasmalemmae (Fig. 5.41) Con A receptor sites on animal cell membranes are thought to be of a glycoprotein nature (Matus, dePetris and Raff, 1973) and it is therefore likely that the Con A binds to elements of the protoplast glycocalyx, as its primary effect.

It is interesting that a possible role for phytohaemagglutinins in the whole plant has been suggested (Hamblin and Kent, 1973). Endogenous phytohaemagglutinin has been implicated in the uptake of bacteria into bean (Phaseolus sp.) roots, facilitating the establishment of a nitrogen fixing symbiotic relationship. This observation, the fact that the same substance will cause the adhesion of red blood cells to root hairs, the animal cell agglutinating capacity and the action of phytohaemagglutinins on lysosomes (Lucy, 1969) indicate that there may be many more, as yet unexplored, actions of Con A on plant protoplasts.

Lysozyme has been cited as a protoplast fusion inducing agent by Potrykus and Hoffmann (1973). However, in the present work, adhesion alone was induced by treatment of protoplasts with lysozyme under the conditions quoted by the above authors. They suggest that lysozyme induces fusion by a "membrane modifying action" involving a mechanism similar to that of the hyaluronidase produced by mammalian male gametes (Austin, 1968). Under the conditions of use, lysozyme will be acting as a polycation (its isoelectric point is at pH 10.7) and it is therefore suggested that the protoplast adhesion involves a bridging effect again. Certainly, extremely low concentrations would be required to allow close membrane proximity. Further clues to the action of lysozyme may be derived from its enzymic action upon mucopolysaccharides and its binding to sugars (Barman, 1969).

Hartmann, Kao, Gamburg and Miller(1973) attempted to induce protoplast fusion by inducing specific agglutination in the presence of complement sufficient serum. The work demonstrated ,not surprisingly , that protoplast surfaces do bear proteins and other antigenic compounds, but the specificity of the (rabbit) sera was negligible. Controlled fusion was not possible. The sera required heat treatment to induce adhesion without lysis. The rather simpler agglutination by Con A would have achieved as much.

The problem of lysis is ever present once a fusion reaction has been initiated. Many of the treatments described in Chapter 5 induced lysis, or produced preparations which, in the electron microscope, indicated that the threshold of complete lysis had been approached. Development of control techniques has permitted the induction of animal cell fusion by treatments which could cause total lysis(Ahkong et al,1972;Bachi and Howe,1972). Protoplast fusion by sodium nitrate treatment can be controlled although it is potentially lytic. However the attempts to control the Sendai virus and lysolecithin effects upon protoplasts were of rather limited success. (Sections 5.3 and 5.4.) Nonetheless it is believed that the potential for control is there.

The problems imposed by working with an osmotically very sensitive system are evident.Potrykus and Hoffmann(1973) have claimed that deplasmolysis aids protoplast fusion and the uptake of large organelles. This is difficult to accept in the light of the magnitude of the plasmalemmar lesions which must be produced in the treatments described.

It has been suggested that the treatment of animal cells with fusion inducing compounds initiates swelling and an associated fluidity of the plasma membrane, which permits a greater degree of contact between membrane phospholipids, thus facilitating fusion.(Ahkong,Cramp,Fisher,Howell,Tampion, Verrinder and Lucy,1973)The action of retinol ,lysolecithin and the lipid emulsions on protoplasts(Sections 5.3.1 to 5.3.3) would suggest that a reaction is occuring which permits osmotic swelling and eventually bursting ,

by affecting plasmalemma permeability. The use of very highly dispersed lipid emulsions might provide a means of controlling the reaction but the treatment would have to involve the use of an adhesion inducing agent. Protoplast adhesion was poor in the most dispersed emulsions used; this is supported by the electrophoretic evidence that oleates, at least, induce no protoplast surface charge modification. (Grout, 1973)

Bala Bawa and Torrey (1971) attempted to induce protoplast fusion using Sendai virus, but were unsuccessful. The work described in Section 5.4 would suggest that the effect of the virus upon the protoplasts depends very much on the particular preparation in use. Those which failed to have any marked effect upon the protoplasts are thought to have deteriorated somewhat in storage. (They were stored at -20°C initially) The observation of direct membrane adhesion, without intervening virions (Fig. 5.48) resembles the early stages of virus induced animal cell fusion (Toister and Loyter, 1973). Unfortunately, although membrane bridges were formed, the protoplasts were in too poor a condition to determine the relationship between the virus and the plasmalemma at the actual points of fusion (Figs. 5.49a and b).

The susceptibility of animal cells to virus induced fusion has been associated with plasma membrane lipid composition, (Choppin, Klenk, Compans and Caliguirri, 1971) but not without criticism (Poste, Reeve, Alexander and Terry, 1972.) It may be relevant that the cholesterol content of plant protoplast plasmalemmae is thought to be negligible, (Korn 1969) implying, according to Choppin et al (1971) a very high susceptibility to viral effects.

6.2.3 Inorganic salt induced effects

Of all the salts used (Table 5.1) sodium nitrate was the only one to produce an unequivocal fusion reaction. The reason for this is not understood, since the electrophoretic effects of many of the salts used were the same as those produced by sodium nitrate. It is possible that there

may be a critical balance between fusion inducing and deleterious effects which, in the case of sodium nitrate, favours fusion.

The rapid formation of very large areas of adhesion would suggest that the entire protoplast surface has the capacity to undergo adhesion, once the necessary contact is established. Within any one region, points of closer adhesion are observed (Fig. 5.6c) suggestive of a higher activity. Poste (1972) has suggested that there may be a relationship between animal cell coat thickness and adhesion/fusion capacity. The removal of surface sialic acid can facilitate animal cell fusion (Ahkong et al, 1973). Therefore it is possible that these points of closer adhesion may represent breaks in the glycocalyx, the scars mentioned in Section 6.1.3. (However Power (1971) suggests that increased glycoprotein material may lead to closer adhesion).

The establishment of points of fusion follows (Fig. 5.27) without any intermediate stages being apparent, although other observations suggest that pentalaminar (Fig. 5.1) or forked (Fig. 5.20b) membrane configurations would be feasible. The points of fusion are separated by parallel plasma-lemma, which implies that all the points of close adhesion have fused. The mechanism by which the actual membrane fusion occurs, can only be speculated upon. In the scheme proposed by Poste and Allison (1971), involving a four stage: contact, induction, fusion, stabilization, process, the first stage is considered capable of triggering the following stages, without the intervention of foreign molecules. Thus the salt induced adhesion may induce instability in the membranes, possibly involving a bimolecular leaflet to micelle transformation (Lucy, 1969). It is possible that the membrane reforms, linking the two plasmalemmae, but higher resolution electron microscopy would be required to confirm this. The limitations upon membrane flexibility might, however, prevent this (Mason and Lee, 1973). It is therefore suggested that the regions of instability could spread, 'eroding' the periphery of the fusion region and eliminating

the unfused membrane. Alternatively, a stretching of the membrane, once its continuity had been broken by the points of fusion, might occur. Redistribution of membrane, such as that which occurs following the initiation of spontaneous fusion, could not very easily occur, since the unfused membranes remain closely adhered. This would not be the case, however, with fusion induced by an agent such as Sendai virus. Figures 6.6 and 6.7 compare the fusion processes.

It was attempted to examine the fusing plasmalemmae using freeze-etching. Satir, Schooley and Satir (1973) have successfully investigated a membrane fusion system in Tetrahymena by this means. However in the case of fusing protoplasts, it appeared to be impossible to obtain the necessary membrane fractures. The fact that urea induces some similar effects to salt treatment, and also alters membrane fracture planes (Necas and Svoboda, 1973) may be relevant.

The various membrane changes formed by salt treatment would imply that a certain degree of membrane fluidity is induced. The formation of plasmalemmar protrusions was the most common effect (Figs. 5.10 to 5.14). Their formation would not appear to be essential to or to invariably lead to fusion, although, according to Poste and Allison, (1972) the enhanced membrane contact facilitated by the radius of curvature of the protrusions would facilitate contact and fusion. It is interesting that no marked osmotic swelling was associated with protrusion formation, especially since auxins, some of which are reported to induce protoplast bursting (Power, 1971; Hall and Cocking, 1971), also induce protrusion formation. (Section 5.2.4)

Although some of the more extreme membrane changes, e.g. Figures 5.19 and 5.20, are thought to be the combined effects of salt treatment and fixation, it is difficult to decide whether others are artifactual or not. The localised intensely staining regions of plasmalemma, observed in Figure 5.18, resemble the 'dense granules' described by Burgess et al (1973b)

Figure 6.6

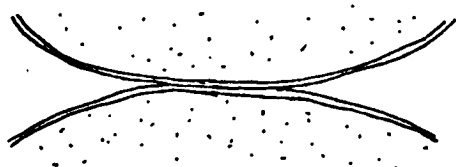
Figure 6.7

e.g. Sodium nitrate

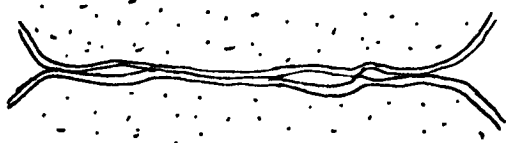


untreated protoplasts

addition of salt



membrane adhesion



membrane instability?

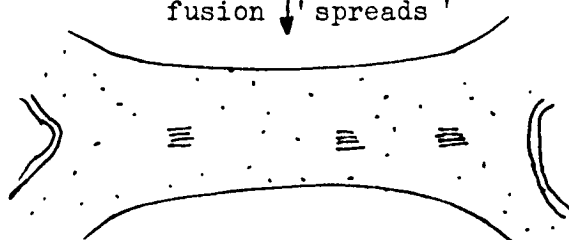


membrane fusion



A. no membrane continuity
B. membrane continuity

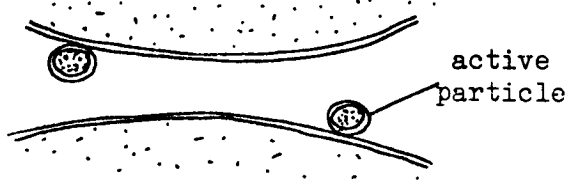
fusion 'spreads'



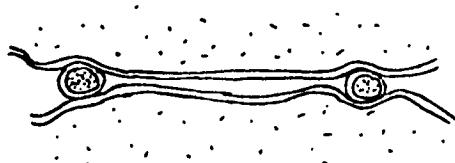
vacuole fusion follows,
first stage is a thinning of
cytoplasm in the fusion region

e.g. Sendaivirus

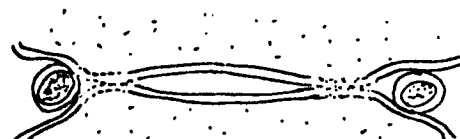
addition of 'virus'



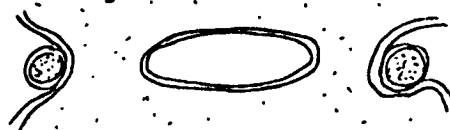
membrane adhesion



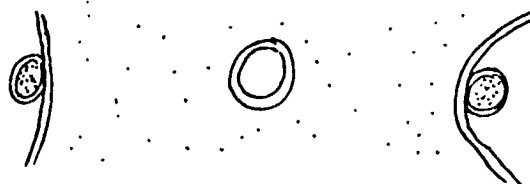
localised membrane activation ?



membrane bridges fusion form



membrane bridges redistribution widen



following stages not
observed, material too unstable

on untreated tobacco protoplast plasmalemmae. The regions of intense membrane activity forming lomasome like structures are substantiated by freeze-etching evidence, (Fig. 5.14) and they resemble structures reported by other workers. (Willison, 1973; Burgess, Motoyoshi and Fleming, 1973a; Mahlberg, 1972). The latter two reports suggest an involvement with uptake phenomena. Willison, Grout and Cocking, (1972) have demonstrated that such plasmalemmar extensions are bounded by stretched membrane. The differential staining effect observed in Figure 5.15 may relate to the condition of the membrane. The majority of the plasmalemma and the underlying vesicles stain deeply whilst the mass of small external vesicles is only lightly stained. Whether the staining is produced by the uranyl acetate stain applied during dehydration, is not known. Wheeler and Baker (1973) have observed a darkening and thickening of membranes exposed to certain heavy metals, suggesting that the staining effect above may be due to the actual salt treatment.

Thus the treatment of protoplasts would seem to produce a whole range of membrane effects, some of which may be inhibitory to fusion. Until the nature of the effects is fully understood it will not be possible to determine why sodium nitrate alone induces fusion.

6.3 FUSION BODIES: THE EARLY STAGES.

6.3.1 The regulation of fusion body size.

Control over fusion body size is desirable for a number of reasons: As mentioned in Section 6.2.1, spontaneous fusion bodies are occasionally too large to handle without damage. The assumption of a spherical form reduces the plasmalemma and tonoplast surface areas per unit volume of cytoplasm to a minimum. This change could, in large fusion bodies, affect uptake, diffusion and sometimes photosynthesis. (See Section 6.4.1). Also, the larger the mass of cytoplasm, the more complex will be its internal relationships compared with those in the single protoplast.

A technique such as electron microscopy which assesses individual structures, can be used as an investigatory technique with heterogeneous preparations. (Section 3.4.3). However in physiological studies assessing general behaviour and growth characteristics, conclusive results can only be obtained from a preparation with some degree of uniformity. The present study has shown that the viability of large fusion bodies is low, an observation supported by Bourgin, Chupeau and Morel (1972), and Woodcock, (1973). Therefore, a preparation in which there is a high frequency of uniformly small fusion bodies may be the ideal experimental material.

The size of an induced fusion body is difficult to regulate. In salt induced fusion, this could necessitate a disruption of the preparation at a critical stage in the fusion reaction. It is difficult to envisage a mechanism which would facilitate size regulation without dispersing all aggregates if applied too early, or cause considerable cytoplasmic damage if applied too late. (Fig. 6.8). It is suggested that some degree of

control might be possible if the initial aggregate size were minimized without inhibiting protoplast contact. Sedimentation onto a large flat surface instead of into a pellet at the base of a tube could possibly prevent the familiar mass adhesion reaction. The use of additional means to maintain adhesion, e.g. by flotation through a dilute Con A solution, (Section 5.2.3), might permit some large aggregate dispersion whilst maintaining small aggregate integrity.

Reactions between protoplasts in aqueous solutions of lysolecithin are thought to be similar to the above. However it is considered that lipid emulsions and Sendai virus produce an entirely different effect, due to their particulate nature. Specific points on the protoplast plasmalemma are 'activated' particularly where units of the active agent lie between protoplasts. The problem of mass adhesion does not appear to be as great. If the fusion reactions could be controlled it is considered that these inducing agents would enable a finer degree of regulation of the dimensions of fusion bodies. Figure 6.9 compares lipid emulsion/Sendai virus induced effects with those induced by salts.

The formation of spontaneous fusion bodies clearly involves a much more easily manipulated reaction. It has been established that spontaneous fusion is brought about by the expansion of plasmodesmata. Therefore treatments to produce desired levels of spontaneous fusion must act upon this mechanism. The range of levels of fusion is clearly extensive, (Fig. 3.192), however to draw definite conclusions about how various levels are induced is difficult. Clearly pretreatments which are likely to break plasmodesmata lower the level of fusion. (Section 3.5.3, Table 3.2). The range of osmotica employed during isolation (Table 3.3) did not appear to be limiting in terms of plasmodesmatal breakage, the higher osmotica tending to produce a higher level of spontaneous fusion. It is suggested

Figure 6.8

THE REGULATION OF FUSION BODY SIZE: SODIUM NITRATE INDUCED FUSION

1. Preparation disturbed too early; protoplasts separate again.
2. Preparation disturbed at intermediate stage; small fusion bodies form.
3. Preparation disturbed too late; fusion is advanced and considerable damage results.

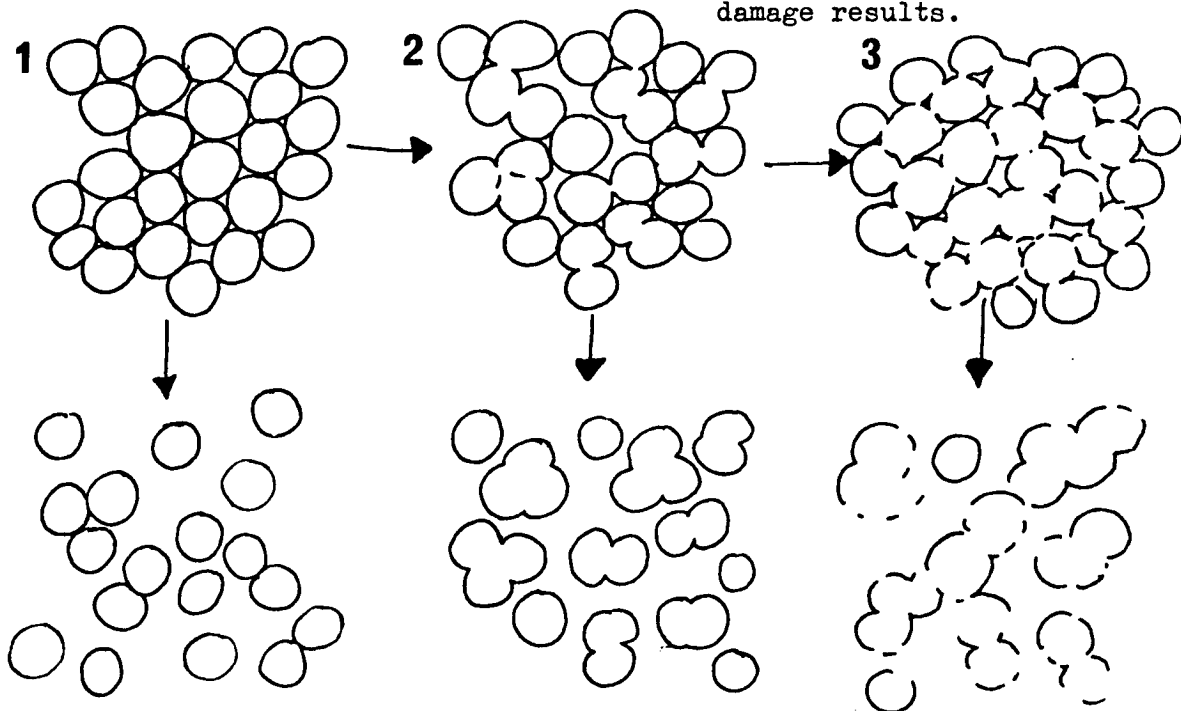


Figure 6.9

THE REGULATION OF FUSION BODY SIZE: FUSION INDUCED BY e.g. SENDAI VIRUS

Here the agent is particulate. Thus at the point of action there is no difference between high and low concentrations of agent. However lowering the concentration does reduce the number of points of action.* In contrast, with sodium nitrate treatment, lowering the concentration simply reduces the overall charge effect. i.e. there is an "all or nothing" effect which is consequently less susceptible to manipulation.

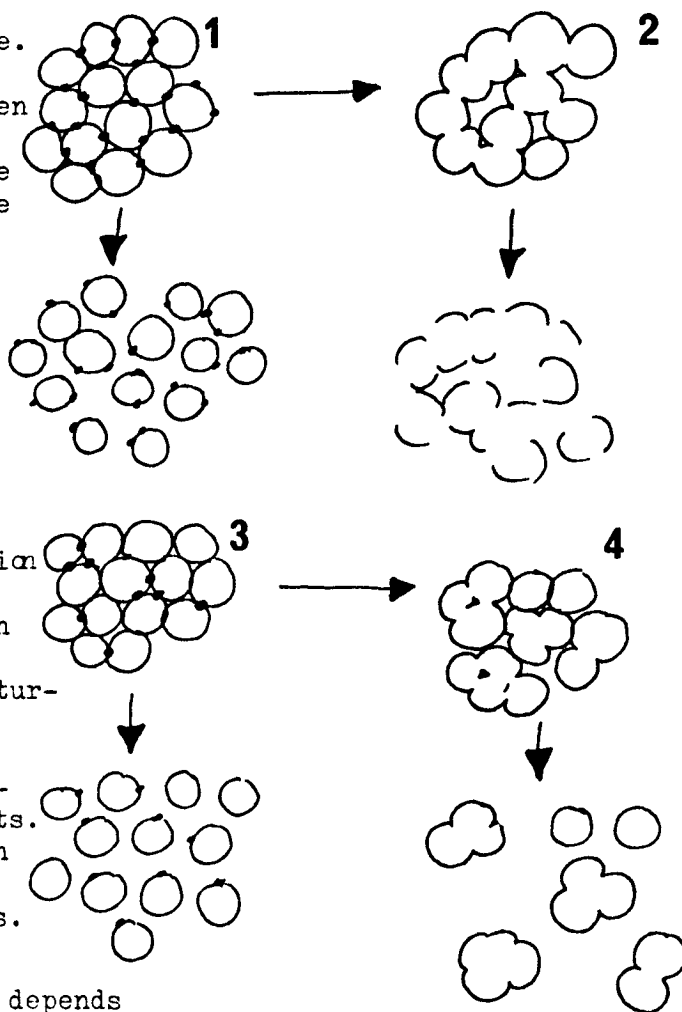
Right:- 1,2. High concentration of agent.

3,4. Lower concentr'n of agent.

1,3. Preparation disturbed too early; protoplasts separate again.

2. Preparation disturbed too late; lysis results.

4. Points of fusion are limited, disturbance separates small fusion bodies.



* Note, success of technique depends nonetheless upon achieving control of the fusion reaction.

that this was due to increased plasmolysis permitting increased enzyme penetration. Most of the enzyme concentrations used, (Table 3.3) again did not appear to be limiting. This may possibly be due to an equilibrium between cellulolytic and deleterious effects being established at any enzyme concentration. (For example, compare preparations 4 and 5, Table 3.3 and Figure 3.192). All that would appear to be required is a degree of wall digestion which permits an overall high yield of protoplasts. The influence of temperature and enzyme purity have already been discussed. (Section 6.2.1).

The relationship between yield and the level of spontaneous fusion does not appear to hold over a range of plant ages. As shown by Figure 3.194, the level of spontaneous fusion as a percentage of yield peaks at approximately 55 days whereas the yield peaks at approximately 70 days, producing a plateau of high absolute fusion levels at 55 to 75 days. (The values for 82 days are thought to be anomalous). This suggests that other factors such as internal osmotic pressure changes and changes in the plasmodesmata (Burgess, 1971) over the range of ages may be critical.

It is considered that the manipulation of the level of spontaneous fusion is readily carried out, but can only be considered empirical. Variations between experiments were frequently as great as variations within experiments, necessitating careful observation of internal controls. In other studies this has not always been done. (Woodcock, 1973). Therefore no far reaching conclusions are drawn from the quantitative data.

Accepting that a high level of spontaneous fusion can be produced, the consideration of fusion body size can be examined. A considerable flexibility is observed in the tobacco leaf protoplast system, fusion bodies forming from two to more than forty contributing protoplasts. However, in all but the low yielding preparations, the fusion bodies are distributed

over the range of sizes. (Figure 3.192). Plots of percentage of uninucleate protoplasts and the more meaningful variable, percentage of nuclei to contributing uninucleates, against the mean number of nuclei per protoplast produce similar curves. (Figs. 3.193 and 6.10) This might appear to be a self evident relationship but as pointed out in Section 3.5.3, it indicates a degree of predictability and lack of flexibility in the system.

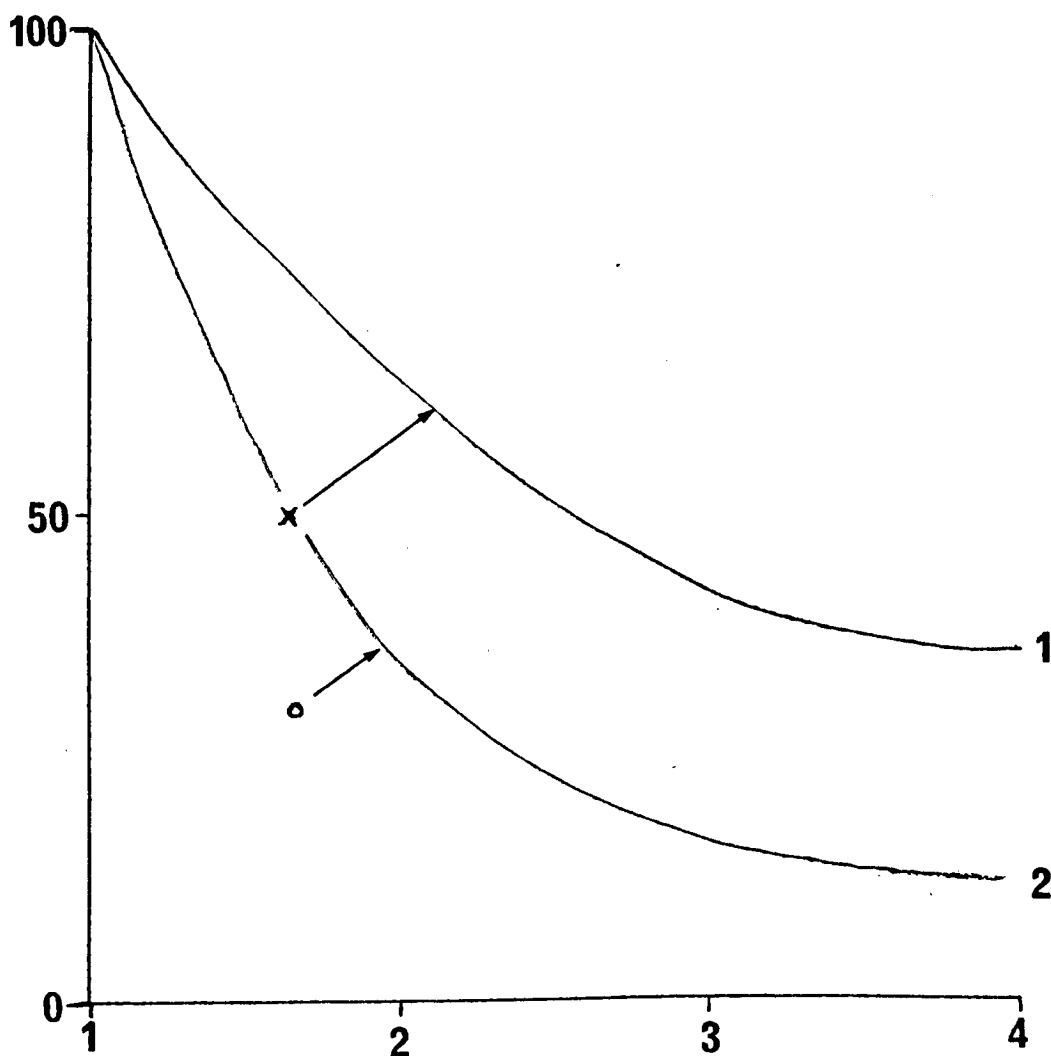


Figure 6.10

Percentage of uninucleates (1) and percentage of nuclei contributing to uninucleates (2) plotted against mean number of nuclei per protoplast. Values relating to a hypothetical preparation are indicated by x and o. (see text). (The arrows indicate the displacement of these points from the relevant curves.)

For example, a preparation containing 50% uninucleates, 40% binucleates and 10% trinucleates is unlikely to be isolated. These figures give values of 50% uninucleates, 31% of the nuclei contributing to uninucleates and a value of 1.6 nuclei per protoplast. The values are plotted in Figure 6.10. Clearly they do not fit the system. Thus, however the material might be manipulated during isolation, no such preparation could be produced.

In addition the graphs (Figs. 3.193 and 6.10) suggest a suitable range within which experimentation upon levels of multinucleation might be conducted. A number of conclusions drawn by Evans, Woodcock and Keats (1973) were derived from data which if presented thus would lie in the near vertical regions of the curves, approaching 100% uninucleation. It is suggested that such results might be more significant if they lay on the near-linear, central regions of the curves.

There remains the possibility of screening protoplast preparations after isolation to select desired fractions. Power (1971) was able to produce a 10% increase in the level of spontaneous fusion bodies by filtering through copper grids and nylon mesh. Woodcock (1973) attempted to enrich fusion body containing preparations by similar means but found that the bi- and tri-nucleates which he wished to separate were remaining with the uninucleate fraction. This is explained by the fact that bi- and tri-nucleates of double and treble the uninucleate volume will have diameters some 1.26 and 1.44 times that of the uninucleate. In a consideration of visual assessment of spontaneous fusion level, diameter differences were considered too low to be used accurately. (Section 3.5.2, Table 3.1). The filtration method could, nonetheless produce reasonably consistent experimental material as long as the presence of uninucleates

were accepted. For example, filtration of the highly mixed preparation No. 12 shown in Figure 3.192 would produce a screened preparation with some 58% uninucleates, 34% binucleates and 8% trinucleates, not very different from the hypothetical preparation discussed above.

The use of buoyancy and drag properties was suggested by Woodcock (1973) as a further means of enriching fusion body preparations, but was unsuccessful. It was attempted, in the present study, to select out spontaneous fusion bodies by selectively bursting the uninucleate protoplasts by deplasmolysis. It was thought that the irregularly shaped fusion bodies might be more resistant to the deplasmolysis. A similar attempt to burst uninucleates by the application of IAA (Hall and Cocking, 1971) was carried out. However, although lowering the osmoticum will induce a more rapid rounding-off, (Section 3.4.2), neither of the means described above was successful in selecting out the fusion bodies. It is suggested that such mechanisms would only work at a critical threshold which was not reached in the treatments applied.

6.3.2. Shape changes in fusion bodies

In fusion bodies formed from highly cytoplasmic protoplasts, there can be few restrictions upon changes of shape. However in highly vacuolated systems (e.g. tobacco mesophyll protoplasts) the vacuole is likely to act as a physical barrier to the cytoplasmic reorganisations necessary to rounding-off. The latter condition was, nonetheless achieved in some induced fusion bodies and most spontaneous fusion bodies.

Two definite patterns of rounding-off were observed, one exclusively found in spontaneous fusion bodies and the other exhibited by some spontaneous but predominantly by induced fusion bodies. (See Sections 3.3.4 and 5.1.5.) The differences between the two patterns lie in the behaviour of the cytoplasm in the fusion region, the degree to which the contributing cytoplasms mix after fusion and the mechanism by which the number of vacuoles in the fusion body is reduced to one.

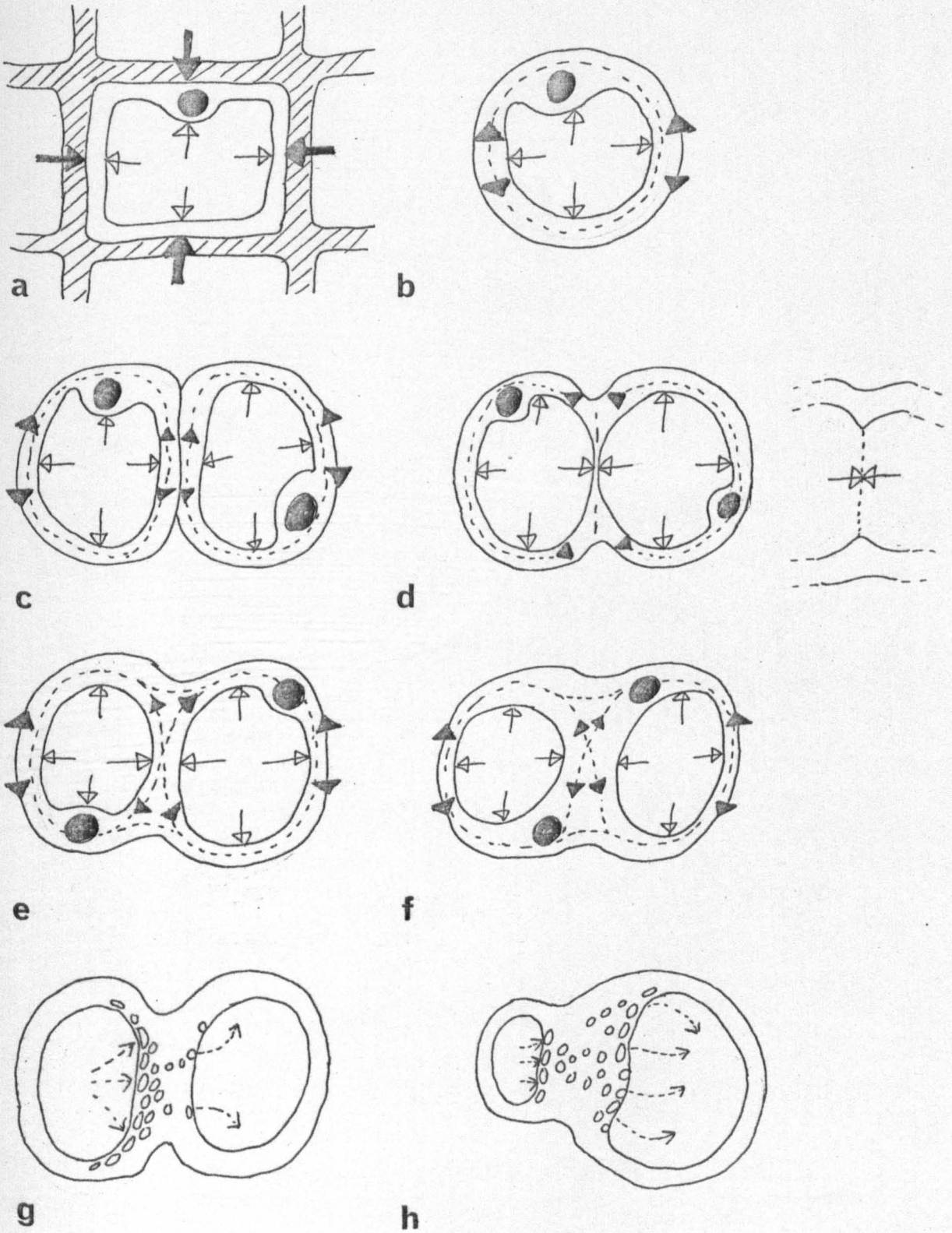
It is possible to rationalise, within limits, the behaviour of fusion bodies by considering a protoplast model in which several antagonistic forces exist: In the turgid tissue, vacuolar pressure is opposed by wall pressure (Fig. 6.11a). The spherical form of the plasmolysed protoplast implies a similar equilibrium between internal vacuolar pressure and forces within the cytoplasm. The extrusion of cytoplasm and vacuole following protoplast damage (Cocking, 1965) and the tendency for lowering of the external osmoticum to induce an exaggerated systrophy and eventual cytoplasmic 'skinning' (own observations) support such a model. Thus a hypothetical system in which vacuolar pressure is contained by cytoplasmic and plasmalemmar elasticity and tension can be proposed (Fig. 6.11b). The tension in the plasmalemma is maintained by membrane continuity, whereas in the cytoplasm it is considered to be a property of the e.r. network.

To apply the model to two protoplasts undergoing induced fusion: At the adhering interface the forces remain at equilibrium (Fig. 6.11c). However, when membrane fusion occurs, the tension in the plasmalemmae would be broken, leaving the insufficient elasticity of the cytoplasm to resist the pressure from the vacuole. It is conceivable that the pressure would lead to a thinning of the cytoplasm in, and a migration of organelles away from the fusion region. After a critical point, the elasticity of the cytoplasms would assist in the process (Fig. 6.11d), vacuolar fusion ensuing. Thus the vacuoles would in a sense appear to aid rounding-off although they do not aid cytoplasmic mixing.

The situation is now contrasted with that of two protoplasts undergoing spontaneous fusion: The membrane reorganization in the fusion region will occur gradually, involving no actual membrane fusion. It is considered that a dynamic equilibrium establishes between the external and internal forces over the period of plasmodesmatal expansion. Fine-structural studies have shown that e.r. continuity and organelle exchange are readily established, thus there is no net depletion of organelles from the fusion region and the e.r. continuity extends the elasticity of the cytoplasm across the fusion region (Fig. 6.11e). Just as cytoplasmic elasticity aided flow away from the fusion region in the former example, it is considered that in this case the elasticity would aid migration towards the fusion region (Fig. 6.11f).

Vacuolar fusion by the mechanism described earlier clearly cannot occur. In Figure 3.85, the presence of masses of small vesicles on the periphery of the vacuoles suggest a second mechanism. The fragmentation of the vacuole into small vesicles which could then be transported through the cytoplasm to a particular location could occur (Figs 6.11 g and h). This again would be a gradual process, resembling a reversal

Figure 6.11 The role of external and internal forces in the behaviour of 215 fusion bodies during rounding-off. (Vacuolar pressure is indicated by open arrows; cytoplasmic and plasmalemmar forces are indicated by solid arrows.)



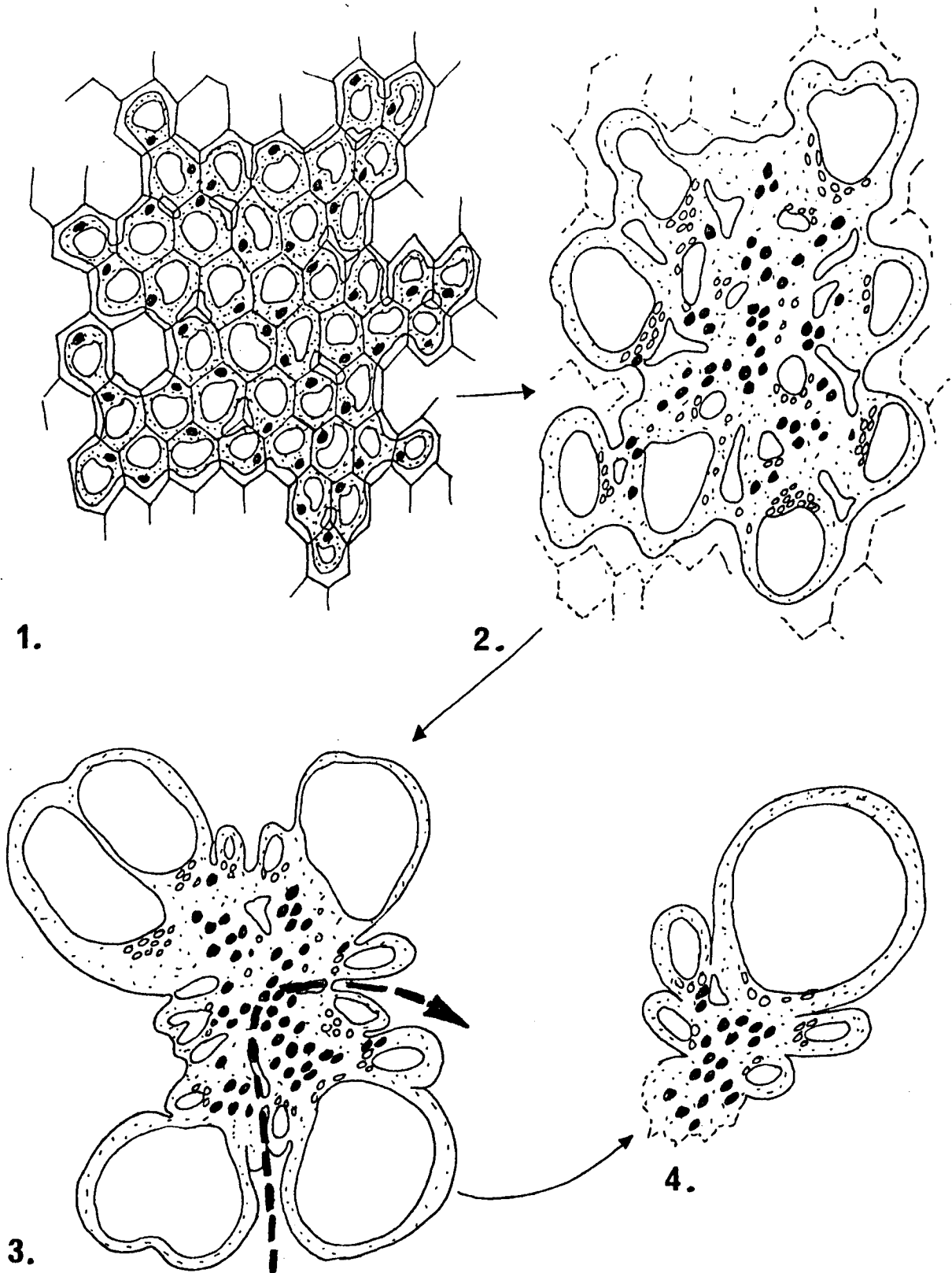
- a. In the intact tissue, turgor is maintained by wall and vacuolar pressure.
b. In the isolated protoplast cytoplasmic and plasmalemmar forces oppose vacuolar pressure.
c & d. Induced fusion: Membrane fusion reduces the resistance to vacuolar pressure; cytoplasm flows from the fusion region; vacuolar fusion occurs.
e & f. Spontaneous fusion: Cytoplasmic continuity enables organelle flow towards the fusion region to occur.
g & h. The transfer of vacuolar space by the formation, transport and fusion of small vesicles. (Direction of flow indicated by broken arrows.)

and then a repetition of the normal mode of vacuolation found in higher plants. Light microscopic observations made by Eriksson (1970) would suggest such a process.

To extend the model to the context of a fusion body forming within the leaf: Clearly, very large areas of tissue are involved in the fusion process and it is unlikely that any one area would be entirely delimited from the surrounding cells. The existence of points of damage on large fusion bodies supports this. Thus, initially, transformations within a much larger area of cytoplasm must be considered (Fig. 6.12, Stage 1). A general centripetal flow of organelles from the periphery of the cytoplasmic mass, with an opposing flow of vacuolar space, would lead to the situation shown in Figure 6.12 (Stage 2). The mechanical forces applied during isolation might permit the intact separation of a small fusion body but a large one such as in Figure 6.12 (Stage 3) would possibly break into smaller units. The units would have some degree of polarity, having one (Figure 6.9, Stage 4) or more major vacuoles and a cytoplasmic region, with an internal organization as in Figure 3.85. A damaged region would be evident.

The inhibition of spontaneous fusion body rounding-off resulting from treatment with Cytochalasin B would suggest that streaming was essential for the rounding-off process (Cytochalasin B is known to inhibit streaming in a number of plant and animal systems (Bradley, 1973)). The application of a lower osmotic pressure is only effective in promoting the later stages of rounding-off. If applied earlier, deplasmolysis is deleterious, suggesting that the initial rounding-off mechanism functions only in the absence of the constraints imposed by vacuolar pressure. In contrast, it is considered that the mechanism of induced fusion body rounding-off, requires little involvement of normal cyto-

Figure 6.12 A mechanism for the formation of large spontaneous fusion bodies.



Stage 1. An area of tissue in which many protoplasts, although plasmolysed, are in contact with neighbouring protoplasts.

Stage 2. Wall digestion is taking place; organelle flow towards, and vesicle flow away from the centre of the cytoplasmic mass, are occurring.

Stage 3. The large fusion body has several peripheral vacuoles, with a massive central systrophy of organelles. The body is susceptible to mechanical damage.

Stage 4. A portion of the fusion body is detached. It exhibits a marked polarity, with a damaged region at the cytoplasmic pole.

-plasmic processes and is merely a response to changes in the various forces involved.

The resemblance between the manner of rounding-off exhibited by some spontaneous fusion bodies, and that common to induced fusion bodies is thought to result from insufficient cytoplasmic continuity being established between the fusing protoplasts. This could be due to poor plasmodesmatal connexions or mechanical disturbance at a critical stage. These suggestions are borne out by the experimental observations.

6.3.3. Nuclear aggregation: a mechanism

The aggregation of nuclei from contributing protoplasts was only rarely observed in induced fusion bodies (Figs 3.34 to 3.36); whereas in spontaneous fusion bodies (including those which rounded-off in the same manner as induced ones) some degree of nuclear aggregation invariably occurred.

Light microscopic observations of various patterns of post isolation behaviour have been reported. Woodcock (1973) describes the aggregation of tobacco (White Burley) mesophyll spontaneous fusion body nuclei. Nuclear fusion is suggested. The micrographs of Bourgin et al (1972) illustrate (without comment, surprisingly) some nuclear pairing in tobacco (W38) fusion bodies. Kao et al (1973) reported the formation of 'giant nuclei' within brome grass spontaneous fusion bodies. None of the above observations have been substantiated by electron microscopy.

The results presented in Section 3.3.4 suggest that in the tobacco (Xanthi) system, at least, a single mechanism of nuclear aggregation is occurring. Some fusion bodies have pairs of nuclei evenly distributed in the cytoplasm (Figs 3.90 to 3.93). Others which are polarised into cytoplasmic and vacuolar regions have a single large aggregate of nuclear material. In the electron microscope, this is revealed to be an aggregate of discrete nuclei, some of which are intimately associated, usually into pairs (Fig. 3.155). The formation of pairs, or fours, precedes that of the aggregate as a whole (Fig. 3.85). The observations described by other workers may simply represent gradations of a similar process.

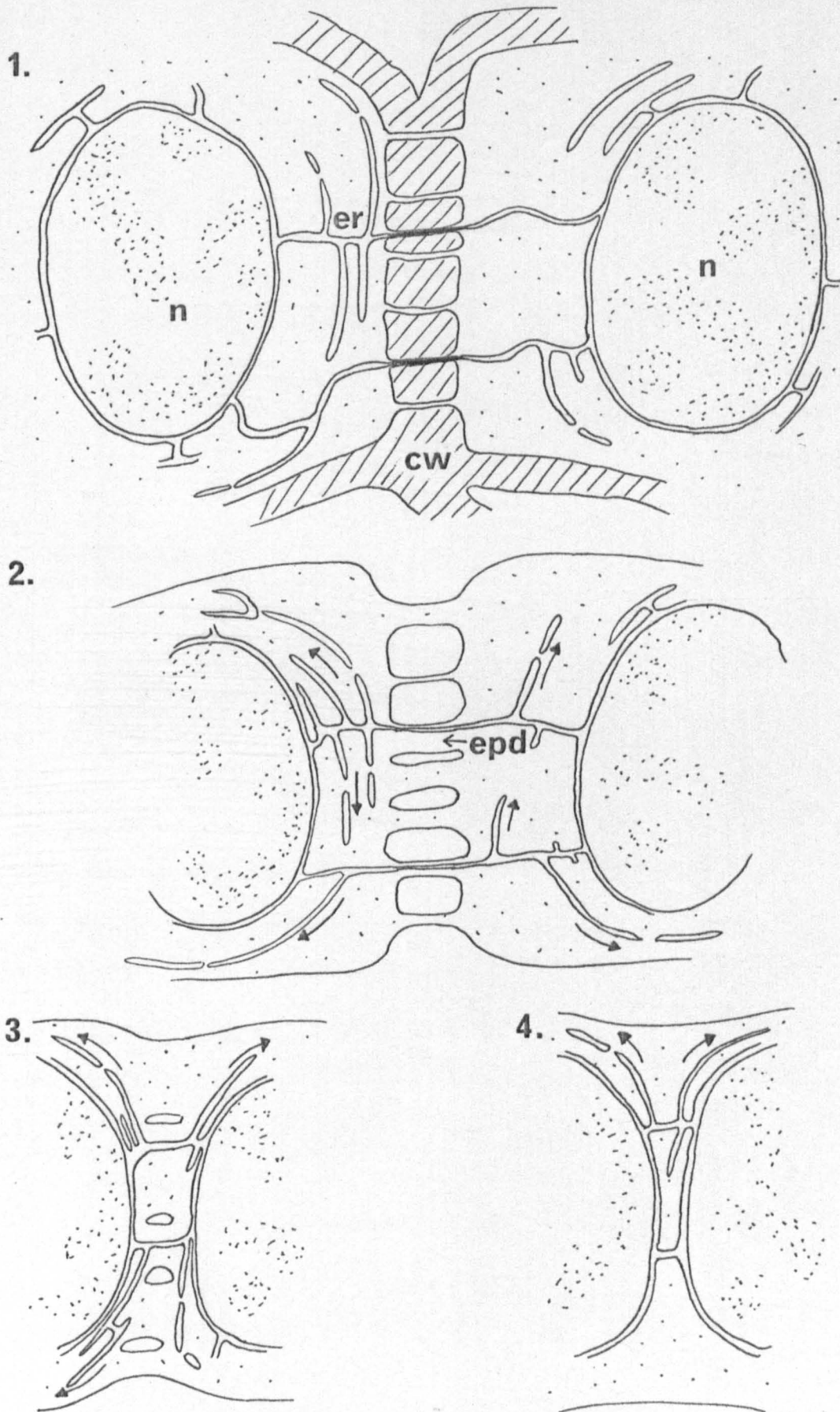
Accepting that spontaneous fusion facilitates nuclear mobility and that organelle systrophy is a common feature of any protoplast system, a certain degree of nuclear approximation could be expected, with

pairing the most likely grouping (on mathematical grounds.) However the frequency of pairing, supported by certain electron microscopic observations, suggest a more specific mechanism.

Continuity of the outer nuclear membrane with the e.r. is an established concept (Robertson, 1964) and the connexion of the e.r. with plasmodesmata is highly likely (Appendix 1; Section 3.1.3.). Thus the nuclei of adjacent cells could have common e.r. connexions (Fig. 6.13, Stage 1.) With the occurrence of spontaneous fusion, the e.r. connexions could draw the nuclei together by a redistribution of the intervening membrane (Fig. 6.13, Stages 2 and 3). Eventually the outer nuclear membranes could become linked by a short membrane bridge. A continuity of perinuclear space is formed (Fig. 6.13, Stage 4.)

There is some electron microscopic support for such a mechanism (Figs 3.156 and 3.162), and it is compatible with the mechanism proposed for spontaneous fusion body rounding-off (Section 6.3.2., above). The virtual absence of nuclear aggregation in induced fusion bodies can be explained by the poor cytoplasmic and therefore e.r. connexions.

The reasons for nuclear pairing in fusion bodies may lie in the relationship of the contributing cells. It is suggested that plasmodesmata become modified with age (Burgess, 1971). It is possible that e.r./plasmodesmatal connexions become similarly modified as cell expansion and wall growth take place. Thus pairs of daughter cells may have more intimate e.r. connexions facilitating the pairing of their nuclei. It is, in effect, a reversal of cytokinesis. However no evidence was obtained for any further processes involving nuclear fusion (other evidence is discussed in Section 6.3.4.).



Stage 1. Nuclear membrane/e.r./plasmodesmatal connexions link neighbouring nuclei in the untreated tissue.

Stages 2 and 3. Wall digestion and plasmodesmatal expansion permit the e.r. connexions to shorten by the redistribution of the membranes.

Stage 4. A stable state is established, with short bridges of e.r. connecting the outer nuclear membranes.

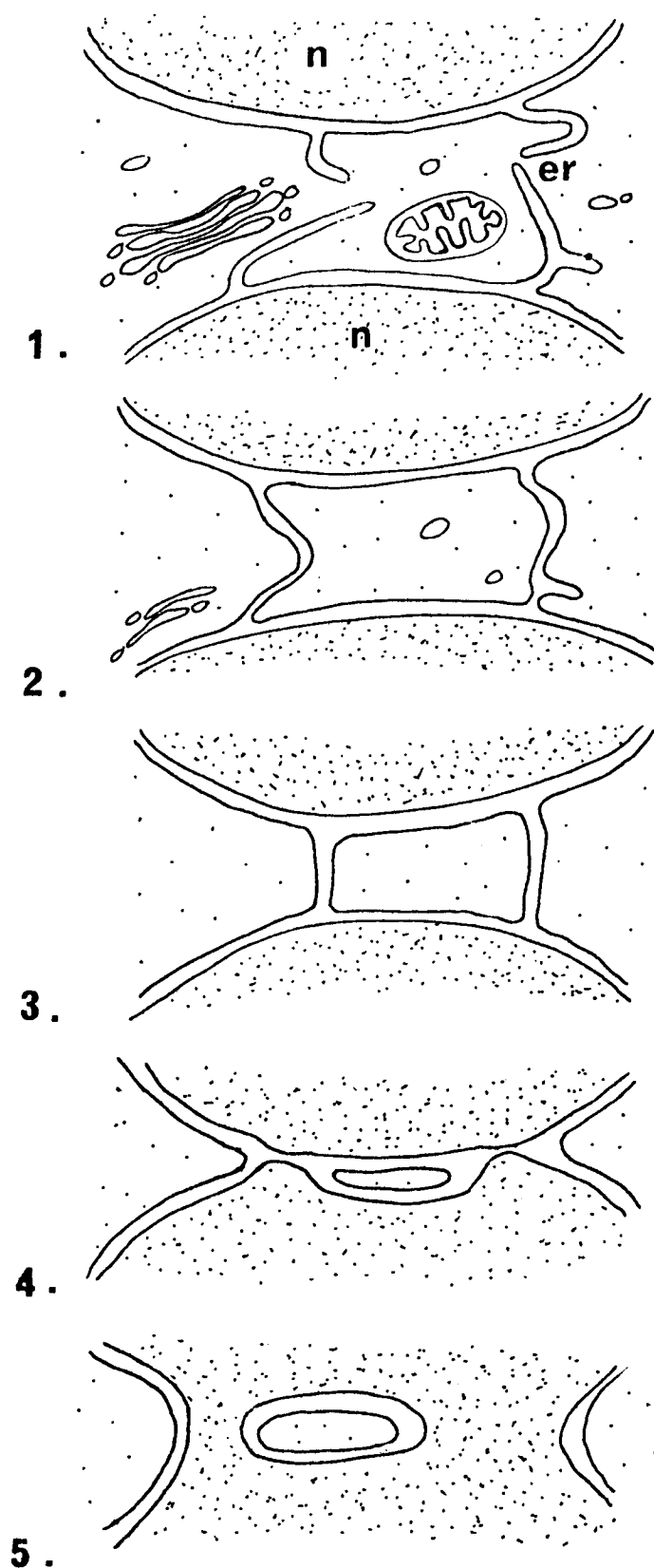
It is interesting to compare the process of nuclear aggregation with the nuclear behaviour during fertilization in higher plants. In the latter, nuclear fusion occurs at three stages, between polar nuclei, between the gametic nuclei and between a second male nucleus and the combined polar nuclei. All three events involve a similar mechanism (Jensen, 1964; van Went, 1970) (Fig. 6.14). The nuclei migrate towards one another, the e.r. membranes fuse and begin to draw the nuclei together. The inner nuclear membranes are eventually brought into contact and they fuse. The nuclear bridges thus formed expand and complete fusion ensues.

The early stages very closely resemble the events occurring during the aggregation of spontaneous fusion body nuclei. This suggests that the eventual nuclear fusion may be feasible in the latter system. The fact that gametic fusion involves an initial fusion of the e.r. membranes further suggests that the mechanisms may be applicable to induced fusion bodies.

The diploid nature of the nuclei in fusion bodies may be of importance in preventing nuclear fusion, as may the fact that the protoplasts are isolated from differentiated cells. In the case of induced fusion bodies, the presence of vacuoles may be an impeding factor. By the use of a haploid system such as tobacco (White Burley) and suspension cultures (Kao et al, 1973), the problems of diploidy and differentiation may be overcome; whilst the use of pollen tetrad protoplasts (Bhojwani and Cocking, 1972) would overcome these two problems and that of vacuolation also.

Nuclear fusion must be considered a pre-requisite to the production of a somatic hybrid. If fusion by the above mechanism fails then a second opportunity may arise during mitosis. Animal cell

Figure 6.14 Nuclear fusion during fertilization in higher plants.



Stage 1. The nuclei move together.

Stages 2 & 3. The e.r. membranes fuse and draw the nuclei closer together.

Stage 4. The inner nuclear membranes are brought together.

Stage 5. The inner nuclear membranes fuse forming inter-nuclear bridges.

synkaryons are formed by such a process (Harris, 1970). Further consideration is given to the problem of nuclear fusion in Section 6.4.3.

6.4 FUSION BODIES IN CULTURE

The culture of uninucleate protoplasts from a variety of sources has been successfully carried out using both liquid and solid media (e.g. Pojnar, Willison and Cocking, 1967; Nagata and Takebe, 1970; 1971). Attempts to specifically culture fusion bodies have met with rather less success. Carlson (1973) has regenerated an apparent somatic hybrid from an induced fusion preparation, using the solid medium plating technique of Nagata and Takebe (1971). The same methodology was employed by Power and Frearson (1973) to study the behaviour in culture of tobacco (Xanthic) spontaneous fusion bodies, with some limited success.

However it is a general observation that induced fusion bodies are extremely difficult to culture under any circumstances, with spontaneous fusion bodies culturing most successfully in liquid media (Sections 3.4.2. and 3.4.3., Miller et al, 1971; Kao et al, 1973). In solid media, spontaneous fusion bodies generally fail to undergo any marked growth or division (Section 3.4.1., Bourgin et al, 1972; Woodcock, 1973). This may be attributable both to the nature of the medium preventing any major shape changes (Fig. 3.108) and to diffusion problems which would be greater in fusion bodies than in uninucleate protoplasts.

The gross and fine-structural changes observed in cultured spontaneous fusion bodies are discussed below, the majority of the observations being of bodies cultured in liquid media.

6.4.1. The cytoplasm

Systrophy is a common feature of the freshly isolated protoplast, but is particularly exaggerated in spontaneous fusion bodies (Figs 3.102a, 3.103a and 3.110). Redistribution of cytoplasmic organelles (Figs 3.102c and 3.111) corresponds with the reversal of plasmolysis changes. The

disappearance of the abnormal peripheral reticulum formation found in spontaneous fusion body chloroplasts (Figs 3.135 and 3.136) is thought to mark a change in photosynthetic rate (Hillard and West, 1971). The systrophied chloroplasts would have caused considerable mutual shading leading to the reticulum proliferation.

Within a short time the chloroplasts begin to function as amyloplasts, storing considerable amounts of starch (Fig. 3.141) (Davey, Frearson, Withers and Power, 1973). Photosynthesis would appear not to be essential for growth in an adequate nutrient medium (Gamborg et al, 1973). The dedifferentiation described by Willison (1973) in cultured tomato fruit protoplasts was observed, but chloroplast budding (Willison, 1973), division (Takebe and Nagata, 1973) or the formation of large lipid droplets (Benbadis, 1972) were not apparent (the latter effect would have been considered a sign of incipient pathological breakdown rather than dedifferentiation (Mlodzianowst and Ponitka, 1973)). Chloroplast and mitochondrial multiplication may have occurred but in the absence of direct observation, verification would require a stereological study.

The apparent 'wound regions' observed in freshly isolated fusion bodies (Fig. 3.85) are not evident during culture. The mechanism by which the cytoplasmic damage is repaired is not known, but the bundles of microfibrils (probably proteinaceous) seen in the region (Figs. 3.185 and 3.187) may be involved in the process.

The presence of dark staining material in the vacuole and cytoplasmic vesicles (Fig. 3.172) is considered to be a result of isolation and culture procedures. However such osmiophilic substances have been reported in whole plant tissues (Burgess, 1972; Willison, personal communication.)

Apart from multinucleation, the presence of cosomes within spontaneous fusion bodies must be considered their most distinguishing feature. Similar cytoplasmic bodies have been reported in tomato fruit locule protoplasts (Cocking and Pojnar, 1970; Willison, 1973,) cereal leaf protoplasts (Pearce, Withers and Willison, 1973) and cultured tomato fruit locule tissue (Pearce, 1972). A similar, but extracytoplasmic structure in tobacco callus cells was reported by Gigot et al, (1972). Cocking and Pojnar (1970) termed the structures autophagosomes, but the fine-structural and histochemical observations of Willison (1973), Pearce (1972) and shown in Figures 3.145 to 3.147 would suggest a relationship between the cosome and the plasmalemma.

All of the systems mentioned above are highly vacuolate and would therefore be susceptible to plasmolytic invagination of plasmalemma (Fig. 6.1), but the direct evidence for this being the mode of formation of cosomes is lacking. In contrast, observations of the stages of spontaneous fusion body formation and rounding-off, point to a definite mechanism for large scale membrane uptake and subsequent cosome formation.

The clefts which lie between lobes of the fusion body (Fig. 3.85) disappear during rounding-off. There is a corresponding appearance of large vesicles in the cytoplasm which contain the membrane bound cytoplasmic fragments originally found in the clefts. (Figs 3.142 to 3.144) Therefore the clefts, rather than opening out, would appear to be pinched-off into the cytoplasm.

The presence in the spontaneous fusion body of large masses of fused cosomes is likely to be inhibitory to the normal cytoplasmic processes. This is supported by the observation that cosome expulsion is generally essential for long term fusion body viability. The small

cosomes which are retained appear inert (Fig. 3.187 h). It is suggested that there may be a minimal functional size, thus explaining why cosomes are not apparent in many protoplast systems which must contain some plasmalemmar vesicles.

Cosome expulsion is frequently marked by the synthesis of large amounts of fibrillar wall material (Fig. 3.154 and 3.188). This may be due to a sudden turnover of membrane, facilitated by the fusion of the cosome membrane with the plasmalemma. Further aspects of cosome activity are discussed below.

6.4.2. The formation of external and internal walls; fusion body division

Cultured spontaneous fusion bodies clearly regenerate an outer envelope which is similar to the cellulose based wall synthesized by uninucleate protoplasts (Grout, 1973). However, in other aspects of behaviour in culture, there are many differences. Fusion bodies exhibit an exaggerated tendency to cytoplasmic budding (Figs 3.112 to 3.119) peripheral cytoplasmic partitioning (Figs 3.106, 3.107 and 3.174) and asymmetrical division (Figs 3.126 to 3.129, 3.185). The lack of uniformity in the preparations makes it rather difficult to draw definite conclusions; however it is attempted, below, to relate some of the phenomena to the abnormal state of multinucleation of the fusion bodies.

The volume of cytoplasm involved in a fusion body is more or less equivalent to that of its contributing cells, but the cytoplasmic distribution is very different. The polarity of the nuclei within the cytoplasm is never completely lost, although there is some reversal of systrophy. Thus any processes under direct nuclear control may decrease with distance from the nuclear pole. For example, cytoplasmic streaming

will be at a minimum at the non-nuclear pole (Yoshida, 1961). The effect would be aggravated if the several nuclei initiated opposing streaming currents (the observation that nuclear associations generally break up would support this.)

The polarised fusion body could be considered to consist of an active highly cytoplasmic syncytium with a virtually inactive vacuolar pole, depleted of organelles. It is hypothesized that many aspects of fusion body behaviour may indicate a process of 'normalization' in the body involving a partitioning of superfluous cytoplasm and a return to uni-nucleation within the syncytium.

Many walls which form within fusion bodies are clearly not related to any form of mitotic division. They are frequently associated with the formation of cytoplasmic buds (Fig. 3.177) or peripheral areas of chloroplast free cytoplasm (Figs 3.106 and 3.174), the former predominating in lower and the latter in higher osmotica and solid media. It is suggested that the interplay of wall synthesis and streaming may lead to these formations. Firstly it is necessary to describe a mechanism for the organized synthesis of cellulose fibrils:

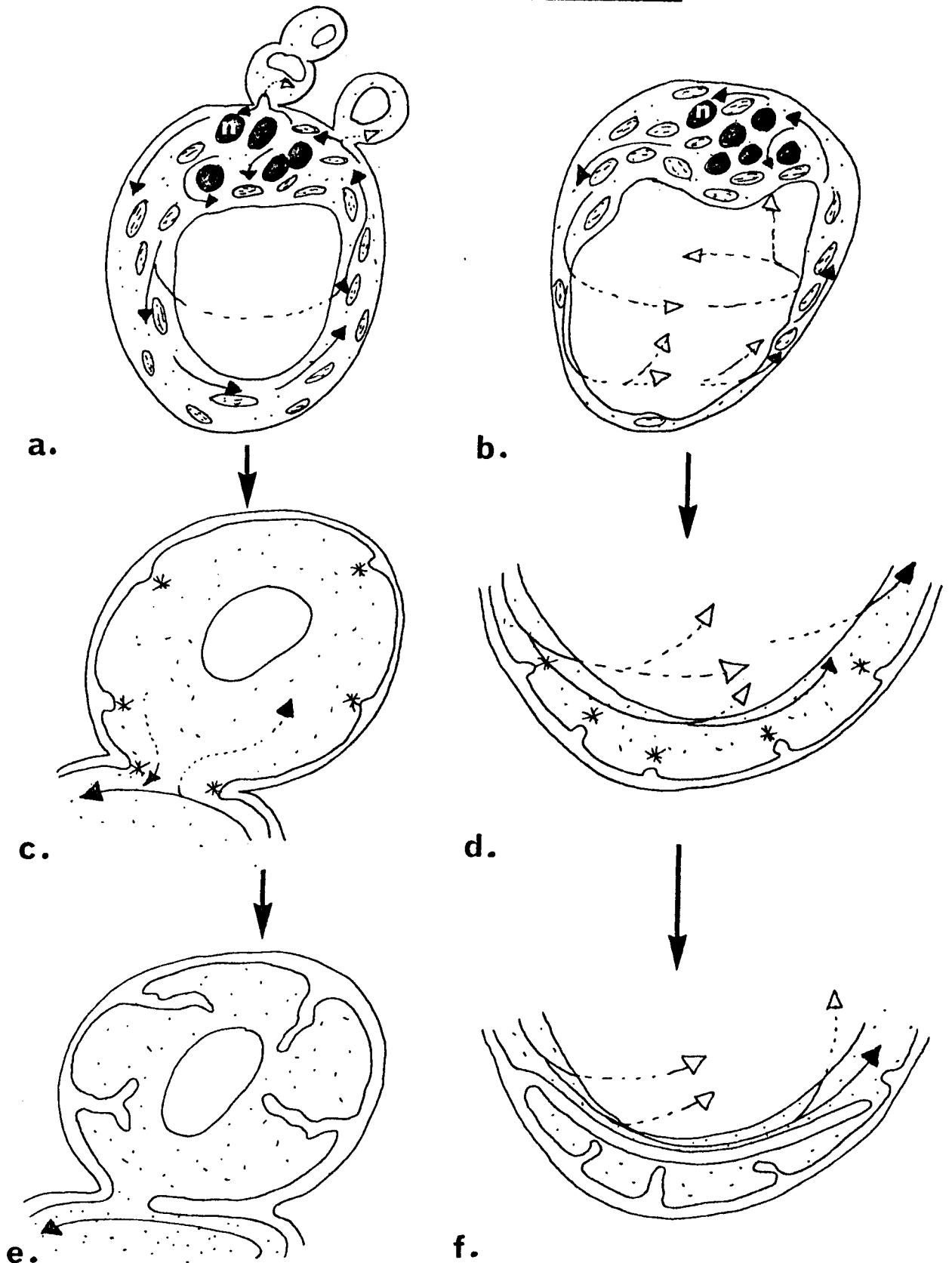
In some plant cells, there is a striking relationship between patterns of cytoplasmic streaming, microtubule orientation and cellulose fibril orientation (van Iterson, 1937; Mühlethaler, 1967). There are several suggestions for the site of cellulose polymerization; most compatible with the present hypothesis is that of mobile sites of synthesis being located on the plasmalemma (Willison, 1973). By combining the two models, streaming currents could direct the movement of sites of cellulose synthesis, thereby orientating the fibril during its formation.

In the nucleated region of the fusion body, the several streaming

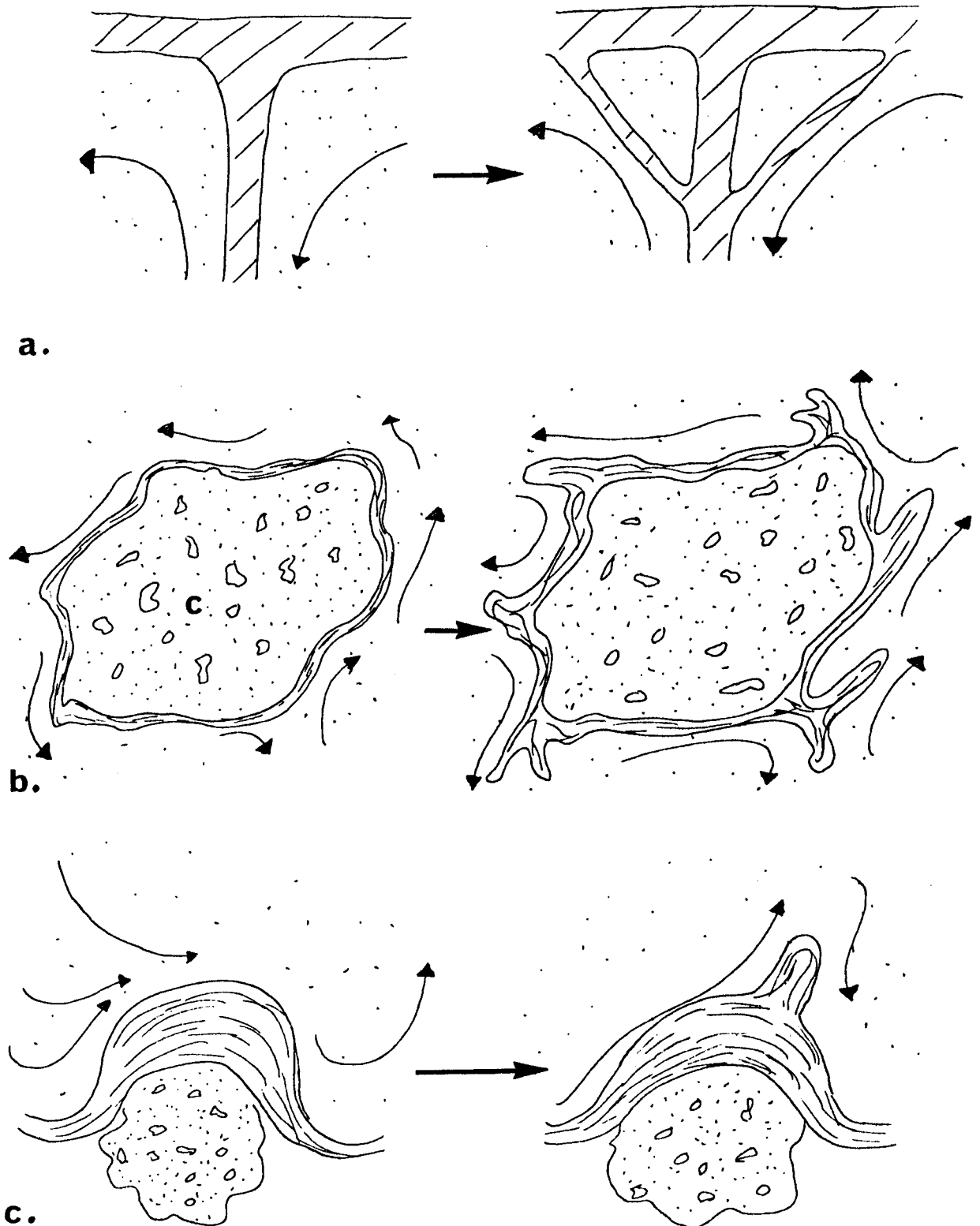
currents could lead to an irregular wall formation. The existence of a high internal pressure could act against weak points in the wall, forming outpushings or buds of cytoplasm. A similar mechanism for bud formation in uninucleate protoplasts has been suggested by Takebe and Nagata (1973). In contrast, Melchers, (1973) considers bud formation to be a prerequisite to normal mitosis in certain systems, the failure of bud formation leading to the failure of mitosis and giant cell formation. This would imply that budding brings about some internal regulation, which is interesting in the present context.

Within cytoplasmic buds, or in somewhat plasmolysed fusion bodies there would be areas of minimal streaming (Fig. 6.15, a and b). The directional influence upon wall formation would therefore be lacking. If stationary sites of cellulose synthesis were to proliferate leading to local irregularities (Fig. 6.15 c and d*) streaming might act upon such sites directing ingrowths of wall material (e.g. Figs 3.174a and 3.177) (Figs 6.15, e and f). A similar mechanism could lead to the partitioning of triangular areas of cytoplasm at cross-wall, outer wall junctions (Fig. 3.180d; Fig. 6.16a) the formation of spurs of wall material on the periphery of cosomes (Fig. 3.151; Fig. 6.16b) and wall ingrowths from partly expelled cosomes (Figs 3.188; Fig. 6.16c).

None of the walls discussed so far have been directly involved with the subdivision of nucleated cytoplasm. Mitotic divisions clearly occur in uninucleate protoplasts (Fig. 3.179) and cannot be dismissed in the case of fusion bodies. Some divisions observed may have been mitotic but were complicated by the presence of more than two nuclei. The type of division wall formation common in fusion bodies, involves the intrusive growth of a furrow which traverses the cell, eventually fusing with the opposite wall. Such a pattern of division is common in highly



- a. Buds extend from the spontaneous fusion body at its nuclear pole. Streaming currents circulate throughout the main body of cytoplasm but are minimal within the buds.
- b. Partially plasmolysed fusion body has areas of minimal streaming at its non-nuclear pole.
- c and d. Details of areas of minimal streaming. Stationary sites of wall synthesis form local irregularities in the outer wall *.
- e and f. The sites at the limit of the influence of the streaming currents could be directed by the currents to form ingrowing walls. The sites thus removed from streaming influence continue to synthesize walls, subdividing the partitioned cytoplasm.



- The partitioning of triangular areas of cytoplasm at cross wall/outer wall junctions. Areas of minimal streaming are thus separated from the main cytoplasmic mass.
- The interference of cosomes with streaming currents could lead to the formation of spurs of wall material extending from the cosome.
- The interference of a partially extruded cosome with streaming currents could lead to the ingrowth of a spur of wall material.

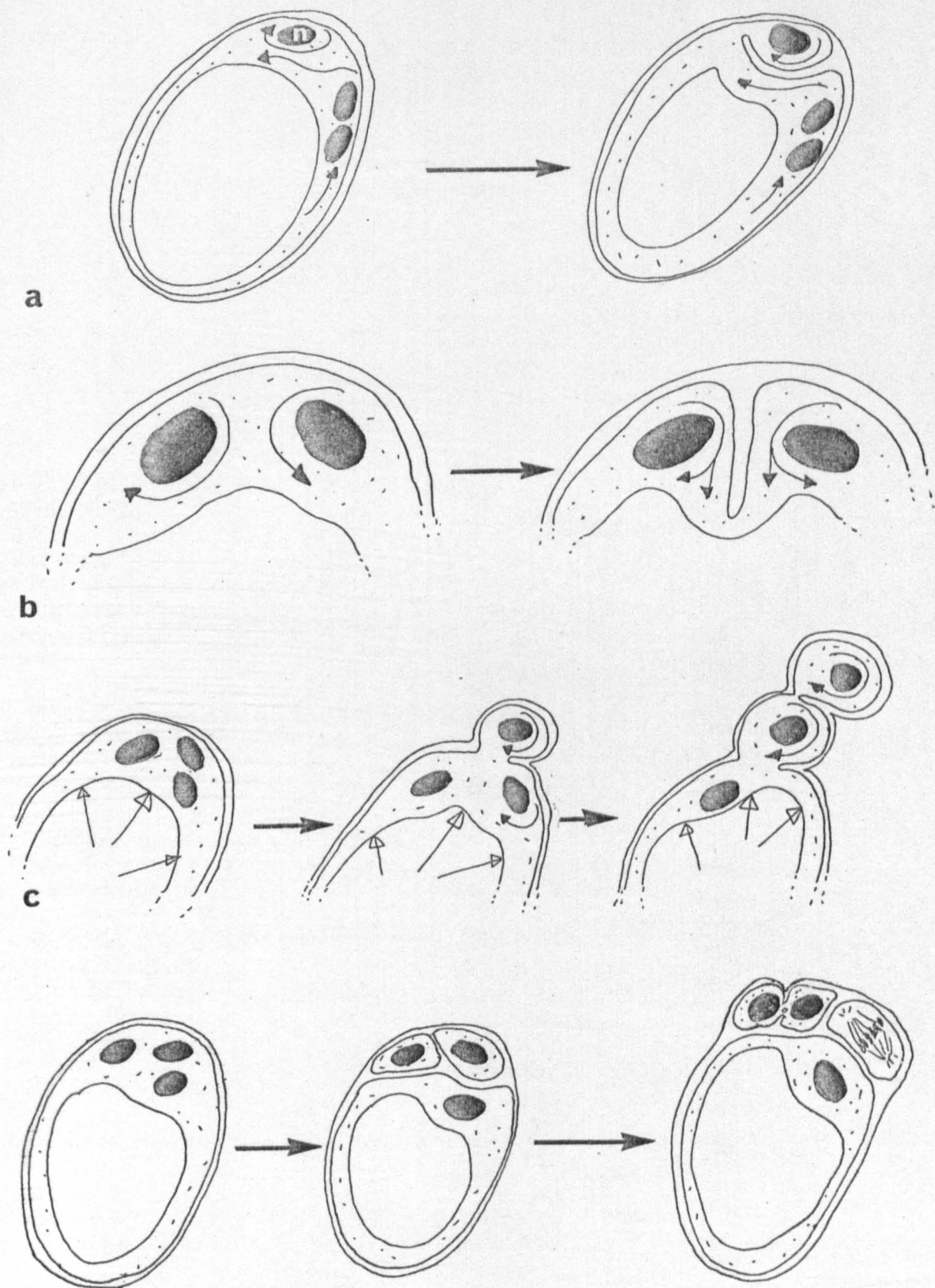
cytoplasmic systems (Pearce, 1972) and may also be characteristic of partially plasmolysed systems (Pearce, personal communication).

It is suggested that in spontaneous fusion bodies the cross wall formation may be occurring under the influence of streaming. There are precedents: Northcote (1973) has described the formation of a cross wall apparently following the direction of a parasitically induced cytoplasmic current. In Chlamydomonas reinhardtii a division furrow forms following the direction of microtubule bundles which are orientated at 90° to the plasma membrane. (Newcomb, 1969).

The partitioning of solitary nuclei (Fig. 3.182; Fig. 6.17a), and the separation of pairs of nuclei (not necessarily daughter nuclei) (Fig. 6.17b) could result from a combination of streaming and wall growth. The observation of the formation of several nucleate buds (Fig. 3.184) is considered to indicate a similar mechanism modified by a higher internal osmotic pressure (Fig. 6.17c). (The inhibition of division in fusion bodies isolated in the presence of Cytachalasin B supports the theoretical aspects discussed).

A number of light microscopic observations suggest that at a certain stage, normal mitotic division may occur either in the cytoplasmic regions of polarised fusion bodies (Figs 3.128 and 3.129) or in those with a central rather than polar systrophy (Figs 3.121 and 3.122). The latter was a very rare observation unlike the former, where partitioning may have established a suitable degree of organization for division to take place. (Fig. 6.17d).

The above observations and evidence from nuclear counting (Section 3.5.4.) point to a return to uninucleation. In the context of the aim to produce a somatic hybrid, this could be advantageous if normality were restored after a nuclear fusion event, but disadvantageous if it



- d**
- The partitioning of a solitary nucleus by the ingrowth of a streaming-directed wall.
 - The separation of two nuclei by an ingrowing wall. The two streaming currents direct the wall.
 - In a low osmoticum the combination of budding and nuclear partitioning could form a chain of nucleated units.
 - An initial subdivision may be necessary for the normal mitotic processes to take place.

prevented nuclear fusion. Electron microscopy gives few clues to the likely events. It is necessary to turn to other sources of evidence.

6.4.3. The loss of multinucleates in culture: does nuclear fusion occur?

Four possible causes of the loss of multinucleation can be suggested:

- 1) Selective degeneration of fusion bodies
- 2) Compartmentalization restoring uninucleation
- 3) The maintenance, or a return of nuclear aggregation,
leading to the misinterpretation of nuclear counting
- 4) Nuclear fusion.

A proportion of all protoplasts degenerate in culture. Observations suggest that in spontaneous fusion body containing preparations, the failure to round-off properly or mortality due to the presence of cosomes lead to the loss of a higher proportion of multinucleates.

Mechanisms whereby nuclear partitioning might occur, restoring multinucleation have been described above. However, without knowledge of any other events occurring, the functional significance of the process is unknown.

It is difficult to decide which of the remaining two processes might be occurring. Microdensitometric analysis can indicate the DNA content of a nuclear mass but will not distinguish between closely associated or fused nuclei (Sections 3.5.2. and 3.5.4.). When applied to two spontaneous fusion body containing cultures, the presence of nuclear masses with very high DNA contents was implied (Fig. 3.195). Accepting that the method of selection was unstatistical and that the criteria for identifying multinucleates may have been suspect, the results still suggest that a large proportion of the 'clumped' fraction nuclei may be aggregated or fused. The proportion is considered to exceed the number

of instances of associated nuclei observed in the electron microscope, thereby implying the occurrence of fusion.

The evidence is clearly inconclusive and further work is required. Electron microscopic investigation of the 'giant nuclei' described by Kao et al, (1973) for example, would be desirable. The karyotype of the regenerated plant might be considered the definitive test of nuclear fusion but as will be discussed in Section 6.5, genome stability is not one of the most consistent qualities of tissue culture. We return therefore to the necessity for direct observation of fusion events. In view of the unsatisfactory aspects of solid medium culture, the use of liquid microculture techniques is suggested (Vasil and Vasil, 1973).

The question must be asked, whether fusion bodies can be expected to undergo nuclear fusion. As already mentioned, there are precedents for nuclear fusion occurring by a mechanism resembling gametic nuclear fusion or by common spindle formation at mitosis. There are, however, precedents for syncytia existing without any further nuclear processes occurring (e.g. in fungal, protozoan and gymnosperm systems).

The system studied by Reinert and Hellman (1973) exhibits a tendency to increase the protoplast nuclear content, although mitosis, often synchronous, is occurring. Motoyoshi (1971) and Kao et al (1973) describe extensive synchronous mitosis, the latter workers claiming to have observed nuclear fusion by an undefined process. The regeneration, by Carlson (1973) of a somatic hybrid plant having a chromosome number equal to the sum of the parental diploids and equal to that of the sexually produced amphiploid, suggest that simple fusion of one nucleus from each parent may have occurred.

Thus a range of patterns of behaviour are observed in protoplast fusion bodies, implying that nuclear fusion, particularly at mitosis is feasible, but requires very specific conditions, a study of which is clearly necessitated.

6.5 PERSPECTIVES

6.5.1 Towards a definition of fusion

Despite extensive work on the fusion of plant protoplasts, a common definition for fusion has yet to be accepted. From the point of view of assessing the efficacy of fusion inducers, the establishment of points of fused membrane might be considered an adequate criterion. Two requirements must be fulfilled, however. Research into the mechanism of fusion in certain animal cell systems has indicated the importance of careful examination, particularly of thin sectioned material. Without the availability of goniometer facilities, the misinterpretation of membrane adhesion as fusion is likely to occur. (Kikuchi, 1972) Good fixation of the material is a second requirement. It is impossible to confirm plasmalemmar fusion unless preservation of unfused membrane is assured. (c.f. Davey and Short, 1973)

Certain 'post-fusion' events are not necessarily indicative of membrane fusion. For example, cell aggregates can undergo the coordinated synthesis of a common wall. (Fig. 5.43; Giles, 1972) Thus, closer examination is essential.

When fusion progresses to include cytoplasmic coalescence, then the above considerations are obviously superfluous. It is, however, necessary to ensure distinction between spontaneous and induced fusion, since cytoplasmic mixing is a common feature of the latter. This highlights the importance of a functional definition for fusion. Whilst spontaneous fusion does not involve actual membrane fusion, the product clearly undergoes the modifications necessary for the fulfillment of the potential of a hybrid. Membrane fusion cannot, therefore, be considered as more than the first stage in a continuum of fusion events. If it is necessary at all to pinpoint a specific process within the continuum, then the establishment of e.r. continuity would probably be the most significant.

6.5.2 Somatic hybridization and the role of spontaneous fusion

In terms of the production of a somatic hybrid plant, spontaneous

fusion is clearly limited. The only possibility for using the mechanism of spontaneous fusion would be in the production of a fusion body from an interspecific chimaeral tissue. This has already been attempted by Giles but without success. (personal communication to P.K.Evans.) The degree of intercellular contact within the tissue will obviously be critical. Burgess (1972) has demonstrated plasmodesmata between the two tissues within the periclinal chimaera Cytisus adami, which might provide the necessary continuity to permit spontaneous fusion.

As an indirect use, observation of the behaviour of nuclei within spontaneous fusion bodies might, by comparison with induced homokaryons, indicate the importance of cytoplasmic continuity in permitting nuclear interactions. It is of course possible that a regenerated spontaneous fusion body would itself be a source of genetic variation, producing perhaps a polyploid plant, or a chimaera of two or more ploidies. Thus spontaneous fusion should not be looked upon merely as a phenomenon associated with protoplast isolation.

6.5.3 The potential of somatic hybridization.

As a tool for the study of cellular differentiation and for genetic analysis, the hybridization of animal cells has been invaluable. However, the limits of the system are (fortunately) reached when a full expression of the morphogenetic capacity of the contributing genomes is considered. In contrast, the development of techniques for the somatic hybridization of higher plants has been with this latter aspect as a prime objective.

The somatic hybrid plant regenerated by Carlson (1973) would appear to represent the two parental genomes without any loss of genetic information. However, many instances of genetic instability, both in somatic tissues of whole plants, and in the products of sexual crosses, have been reported. The cross between two species of Hordeum produces a diploid which reverts to monoploidy by the loss of the chromosomes of one parent only. (Jensen, 1973)

Within tissue cultures, genetic variations are common and tend to increase in frequency with repeated sub-culturing.(Sunderland, 1971)

These factors would indicate that the production of a particular somatic hybrid is an extremely unlikely event, becoming less likely with increased diversity of parental material. They do, however, suggest that there is a great potential for variation within the system. Exploitation of the variation and the instability which produces it, could result in the development of analytic techniques paralleling those used in the field of animal cell hybridization. Thus the production of a somatic hybrid plant might be looked upon as just one possible use of the technique of somatic hybridization. This broader approach leads to a consideration of the other means by which genetic variation might be introduced using plant protoplast material:

6.5.4 The potential of the protoplast system.

The naked protoplast is capable of endocytosing macromolecules from various sources. The uptake and multiplication of T.M.V. by tomato and tobacco protoplasts have been demonstrated, (Cocking and Pojnar, 1969; Coutts, Cocking and Kassanis, 1972) indicating that the protoplast has the capacity to duplicate foreign R.N.A.. More recently, reports have been made of the introduction of both bacterial D.N.A. (Ohyama, Gamborg and Miller, 1973) and higher plant D.N.A. (Hoffmann and Hess, 1973) into protoplasts. As yet it is not known whether the nucleic acid is multiplied.

To extend the scheme to include other sources of genetic information: The uptake of bacteria (Davey and Cocking, 1972), isolated nuclei (Potrykus and Hoffmann, 1973) and chloroplasts (Carlson, 1973) have all been carried out with some degree of success. A second means of uptake of cytoplasmic organelles, (permitting a greater degree of mixing since there would be no enclosing membrane), would be by the fusion of enucleate and nucleate units, returning us, in a sense, to the original technique discussed.

Thus the fusion of isolated protoplasts must be considered as only one

of several means of exploiting the potential for genetic manipulation within the protoplast system.

Post script

"A long time ago, so one story goes, two plant breeders tried to cross cabbage with radishes in hopes of getting a plant with edible roots as well as leaves. They succeeded in making the cross and the new exotic species grew vigorously. It was a disappointing attempt, however. The new plant produced the leaves of a radish and the roots of a cabbage.".....John Gerstner,The Furrow

July/August 1972.

SUMMARY

with conclusions

1. Two types of protoplast fusion were studied, namely spontaneous and induced fusion. The former occurs during enzyme digestion and protoplast release, whilst the latter involves the fusion of originally separate protoplasts. In the latter case, substances termed inducing agents are employed to induce the fusion.
2. Protoplasts and spontaneous fusion bodies were isolated from a number of tissues: tobacco leaf, oat root tip, maize root tip, maize seedling first leaf and coleoptile, and onion root tip and leaf.
3. The majority of the above tissues are from monocotyledonous plants, the exception being the tobacco leaf. A common pattern of spontaneous fusion body production was observed in the former, the tissues tending to produce linear fusion bodies. These reflect the parental tissue structure.
4. The process of enzyme digestion of the oat root tip was followed in the electron microscope. It was observed that whilst some plasmodesmata were broken and the protoplasts damaged, many plasmodesmata resisted plasmolysis damage and were able to expand as wall digestion occurred. This expansion permitted the transfer of organelles and cytoplasmic coalescence.
5. The tobacco leaf is structurally very different from the above and accordingly shows a different pattern of enzyme digestion. The spongy and pallisade mesophyll tissues produced spontaneous fusion bodies which differed in size and form and again reflected the parent tissue structures. Light and electron microscopic observations once more suggested plasmodesmatal involvement.
6. It was possible to manipulate the level of spontaneous fusion in the tobacco leaf by altering isolation conditions. Generally treatments which accelerated wall digestion whilst minimizing plasmodesmatal

breakage were conducive to fusion. However a clear interpretation of the results was not possible.

7. The plasmolysis which accompanied enzyme treatment permitted uptake of extracytoplasmic material. Thorium dioxide, included in the enzyme incubation medium became incorporated into cytoplasmic vesicles.

8. The processes leading to , and accompanying the assumption of a rounded form by spontaneous fusion bodies were followed in the light and electron microscopes. The bodies demonstrated two modes of rounding off: The first involved direct vacuolar fusion and the formation of a large vacuolate structure with evenly distributed cytoplasm. The second was rather more complex and resulted in the formation of a polarised body in which the cytoplasm formed a large mass adjacent to one (or more) vacuoles bounded by a thin layer of cytoplasm. Within the cytoplasmic mass, the nuclei were associated, frequently in pairs. Osmotic manipulation could control the rounding off process. Treatment with Cytochalasin inhibited rounding off.

9. Tobacco mesophyll spontaneous fusion bodies were cultured in both liquid and solid media and were compared with similarly cultured uninucleate protoplasts. The latter divided and formed colonies in both media. The fusion bodies failed to undergo any division in solid media, eventually degenerating. In liquid media, the fusion bodies underwent some similar changes to the uninucleates, but were exceptional in their formation of numerous buds and their pattern of internal wall formation. Few divisions could be classified as mitotic, although the partitioning of nucleate as well as enucleate cytoplasmic units occurred. Few clear nuclear associations were observed in the later stages of culture, except in fusion bodies undergoing division. No nuclear fusion was observed. Cosomes were observed in virtually all spontaneous fusion bodies. Many were eventually expelled, those remaining, forming surrounding

layers of wall like material. Degenerating fusion bodies often contained numerous cosomes. The level of multinucleation in spontaneous fusion body containing cultures was observed to decline with time. Microdensitometric assay indicated that the nuclear behaviour was rather more complex than apparent from electron microscopic evidence or nuclear counting. No long term culture of spontaneous fusion bodies was achieved.

10. The induced fusion of protoplasts was studied using several protoplast systems. Proplasmolysis and the use of light and electron microscopic markers enabled the distinction between spontaneous and induced fusion. However, only intraspecific (tobacco, Xanthi) fusion was observed.

Sodium nitrate was the only inorganic salt to induce fusion, although many others induced protoplast adhesion and other plasmalemmar effects. In sodium nitrate treatment, membrane adhesion was followed by localised membrane fusion and organelle migration. The fusion bodies eventually rounded off in a similar manner to the first type of spontaneous fusion body rounding off (8, above.)

11. Lysozyme and Concanavalin A induced protoplast adhesion, the latter particularly strongly.

12. Lysolecithin in aqueous solution caused protoplasts adhesion and lysis. The reaction was controlled somewhat by the use of lipid emulsions containing lysolecithin, but successful fusion was not achieved. Retinol treatment induced protoplast expansion and eventual bursting, with no adhesion stage.

13. The treatment of protoplasts with Sendai virus induced adhesion and lysis, with a likely intervening stage involving membrane fusion. Again the reaction could not be adequately controlled. The condition of the virus was important in determining its effect.

14. The results are discussed in five sections:

- i. Protoplast isolation
- ii. Protoplast interactions
- iii. Fusion bodies: the early stages
- iv. Fusion bodies in culture
- v. Perspectives

i. It was attempted to define the protoplast, drawing on a knowledge of the parent tissue and the isolation process. It was concluded that protoplast isolation results in a considerable modification of the protoplast, in terms of organelle distribution, cytoplasmic concentration, the condition of the plasmalemma and certain physiological aspects. Characteristics of the plant cell which depend upon symplasm continuity are lost. Extracytoplasmic substances, possibly deleterious, are likely to have been taken up.

ii. Protoplast interactions were discussed in relation to spontaneous fusion and induced protoplast adhesion, fusion and lysis. Possible mechanisms for spontaneous fusion were discussed and it was concluded that plasmodesmatal expansion is the primary mechanism for the process. The various examples of protoplast adhesion, fusion and lysis reported by other workers were discussed in the context of the results presented here. It was concluded that adhesion is a necessary prerequisite to fusion but need not necessarily lead to fusion. Also, the lytic action of many agents may involve a fusion process which could be exploited by controlling the sequence of events. The mechanism of sodium nitrate induced fusion was discussed.

iii. The control of fusion body size was discussed. In the case of spontaneous fusion, the level of fusion can clearly be manipulated but the same does not apply to the size of the fusion bodies. Methods for overcoming the problem of fusion body size and lack of uniformity in preparations were considered. In the case of induced fusion, the potential for size control is considered to be present once the actual

fusion reaction can be controlled. Shape changes in fusion bodies were discussed. A model involving antagonistic forces in the the vacuole and cytoplasm of the fusion body was proposed. A mechanism for the aggregation of nuclei within spontaneous fusion bodies was proposed. The process resembles that occurring during gametic fusion. The former process does not proceed to completion. Possible reasons for this and means of overcoming the impediments to complete nuclear fusion were discussed. The potential for such a mechanism operating in induced fusion bodies was discussed.

iv. It is a common observation that spontaneous fusion bodies fail to survive long term culture in solid media; possible reasons were discussed. The gross and fine structural changes occurring in spontaneous fusion bodies cultured in liquid media were discussed and compared with uninucleate protoplasts. The processes of cosome formation and expulsion were discussed. An origin for cosomes was proposed, involving the large scale internalization of plasmalemma during fusion body rounding-off. A mechanism involving the interplay of cytoplasmic streaming and cellulose synthesis was proposed to explain the irregular pattern of internal wall formation in spontaneous fusion bodies. Possible mechanisms whereby the level of multinucleation in cultures could be reduced were discussed. It was concluded that whilst there are precedents for nuclear fusion occurring, it is by no means an inevitability.

v. A functional definition for protoplast fusion was discussed. The concept of a continuum of fusion events rather than a single event was proposed. The relevance of spontaneous fusion to somatic hybridization studies was discussed. It was concluded that, among other things, a study of this type of fusion could aid an understanding of the behaviour of induced fusion bodies. The scope of induced protoplast fusion was discussed in the context of the applications of somatic hybridization and the possible means of introducing genetic variability into plants.

15. Three appendices are included:

1. Aspects of plasmodesmatal structure, as revealed by a study of plasmodesmatal expansion.
2. A consideration of protoplasts isolated from cereals and tobacco epidermal protoplasts.
3. Details of statistical techniques used; the raw data from which the data included in the results sections were derived.

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APPENDIX 1.

Plasmodesmatal Ultrastructure

Arisz (1969) considers plasmodesmata to be essential components of the symplasm, as intercellular junctions facilitating transport within the tissue. Juniper and French (1970), implicate plasmodesmata in the transmission of geotropic responses in roots. Other workers, however, look upon plasmodesmata as passive structures, remnants of events occurring during cell division. (López-Sáez, Gimenez-Martín and Risueño, 1966). There is a similar diversity of opinion concerning the origin of plasmodesmata and their structure in differentiated tissues.

The cytoplasmic nature of plasmodesmata has long been confirmed, (Meeuse, 1957), and there is no doubt that the membrane lining the plasmodesma is continuous with the plasmalemma of the protoplast. (Robards, 1968). Further aspects of fine structure remain the subject of controversy.

There is considerable evidence for plasmodesmatal formation during cell division (e.g. Barlow and Juniper, 1969, O'Brien and Thimann, 1967). However some plasmodesmata do form in non-division walls. (Burgess, 1972). The core of the plasmodesma, termed the desmotubule (Robards, 1968) is thought by many to be of e.r. origin. (Fig. A.1.1) (Porter and Machado, 1960, Lopez-Saez et al, 1966, Frey-Wyssling and Muhlethaler, 1965). The e.r. is intimately involved in cell plate formation and its continuity with the desmotubule is seen in late stages of the process. (Buvat, 1969).

Spindle microtubules are equally in evidence during division and it is suggested by O'Brien and Thimann (1967) and Robards (1968*) that the desmotubule represents a trapped spindle fibre. In the latter model* this

lacks any continuity with the e.r. although possibly in contact with it. (Fig. A.1.2). The evidence for microtubule-like subunit formation in both the tubule and the surrounding plasmalemma, derived using the Markham rotation technique (Markham, Frey and Hills, 1963) is questioned. (see also Helder and Boerma, 1969). The model lacks flexibility literally and in application. It is not applicable to complex plasmodesmata (e.g. Fig. 3.60), it is not consistent with a concept of intercellular communication and in the present context is not compatible with the observations of plasmodesmatal expansion. (Section 3.1.3).

A more recent development of the model (Robards, 1971), reconciles some of these problems. The desmotubule, originally a membrane in a bimolecular leaflet form transforms to a micellar form, (Lucy, 1970) in the late stages of wall formation. The proportions of lipid and protein micelles change. The modulation involves an increase in the particulate protein component. The changes result in the formation of a structure resembling a microtubule. (Fig. A.1.3). Robards reconciles this with his earlier model (1968) by bringing in a concept of a close relationship between structural proteins within the cell. However it is difficult to accept the consequent claim (Robards, 1971), that the distinction between a desmotubule being of microtubule origin or e.r. origin becomes merely a semantic one. Helder and Boerma (1969) propose a much simpler but related model involving a membraneous desmotubule with a micellar structure and the same proportions of constituents as the original membrane.

The latter authors point out that difficulties in section orientation and electron microscope image interpretation have hampered investigations. For example, unless the section passes medianly, the plasmalemma appears to seal the ends of the plasmodesmata. Similar

difficulties apply to visualizing e.r./desmotubule junctions in this constricted area. By digesting away the cell wall, the constrictions it imposed on the plasmodesma are removed. Plasmolysis accompanying wall digestion draws the plasmalemma away from the cell wall. At this stage the strength of the e.r. attachment to the desmotubule, whilst under stress, is clearly indicated. (Fig. 3.20, Burgess, 1971). This attachment is maintained throughout wall digestion. The desmotubule structure becomes clearer (Fig. 3.21) with no evidence of the central core described by Robards (1968). (Fig. 3.22). (It is suggested that this may possibly be a negative staining artifact). Continuity between e.r. and desmotubule is confirmed as expansion ensues. The lateral location of the desmotubule in the widely expanded plasmodesma (Fig. 3.24) would imply some attachment of the desmotubule to the plasmalemma. However the fact that expansion is not hampered and the observations of desmotubule displacement (Fig. 31) indicate that this attachment is highly flexible.

A slight desmotubule expansion is observed (Fig. 3.23 c.f. Fig. 3.21). although this may merely represent a clarification of structure. There is no evidence for further expansion leading to a loss of the narrow tubular structure. One would expect the micellar formed desmotubule suggested by Helder and Boerma (1969) to expand once wall constrictions had been removed. However, the membrane flow necessary for desmotubule expansion must be rather more complex than that involved in plasmodesmatal (plasmalemma) expansion and could therefore, presumably, be latent.

A possible explanation of past difficulties in visualizing desmotubule e.r. relationships may be due to a dynamic situation existing, with the e.r. generally in a bilayered form but occasionally assuming a micellar structure near the end of the (micellar) desmotubule.

The dynamic equilibrium between sealing of the e.r. and continuity with the desmotubule could be altered once wall constrictions were removed thus favouring the latter state, i.e. desmotubule/e.r. continuity.

(Fig. A.1.4)

The final possibility remains of a microtubule of spindle origin being attached to e.r. membranes. Robards (1971) discusses the feasibility of such an association and gives a number of examples of different degrees of association between e.r. and microtubules in animal systems. Additionally, some similar associations are found in plant systems. (Burgess 1970, Pickett-Heaps and Northcote, 1966). Until conclusive evidence is presented to the contrary, this must be considered as a possible explanation of observations of e.r. desmotubule continuity.

In conclusion, the observations of plasmodesmatal expansion are considered to provide some supporting evidence for the models of plasmodesmatal ultrastructure proposed by Helder and Boerema (1969), and Robards (1971) although obviously no one model can satisfy all the types of plasmodesmata observed in plant tissues.

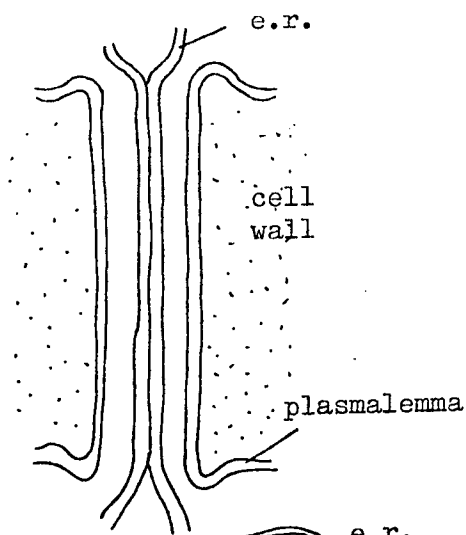


Figure A.1.1 Plasmodesmatal ultrastructure according to Lopez-Saez et al. (1966). The tubule passing through the plasmodesma consists of trapped e.r. membrane.

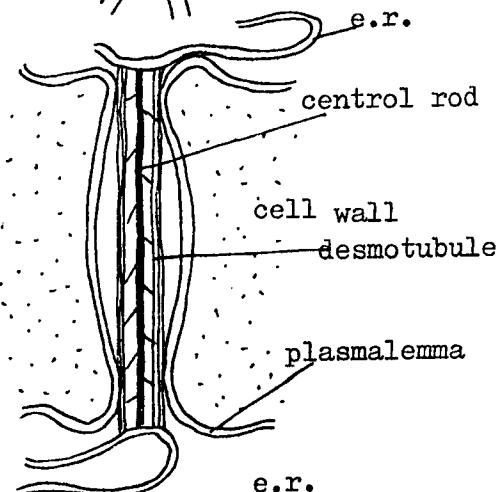


Figure A.1.2 Plasmodesmatal ultrastructure according to Robards (1968). The e.r. is in contact with the desmotubule but not continuous with it. The desmotubule has a dark staining core - the central rod.

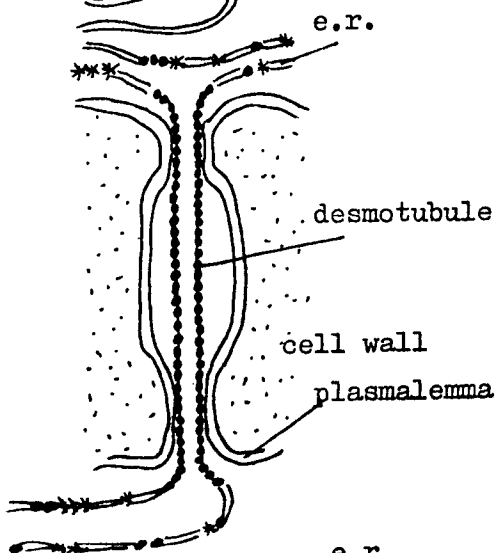


Figure A.1.3 Plasmodesmatal ultrastructure according to Robards (1971). The e.r. is continuous with the desmotubule. The latter is formed from lipoprotein membranes in which the lipid and non-particulate protein have been replaced entirely by particulate protein. The e.r. is shown as alternating bilayers and micelles.

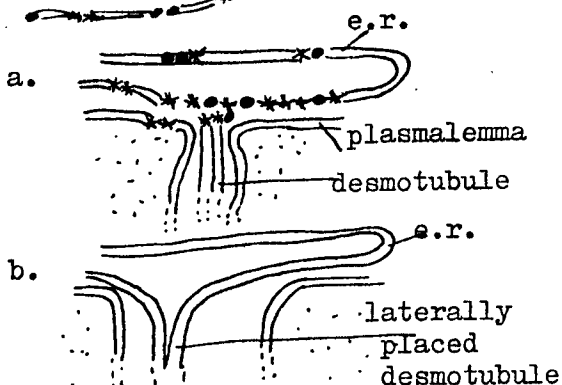


Figure A.1.4

- The e.r. membranes are in a micellar form near the end of the desmotubule.
- Plasmodesmatal expansion favours continuity between the e.r. and the desmotubule.

APPENDIX 2.1

Protoplasts from cereals.

The cereals are crops of obvious commercial importance and means are constantly sought for improving crop yields. Attempts have been made to somatically hybridize cereal protoplasts by the fusion of isolated protoplasts but a constant problem is that of visually distinguishing between protoplasts of different species. (Evans, Woodcock and Keats, 1973). Below is described a marker system using fine structural features of the cereal protoplasts.

The majority of cereals use the Calvin type of carbon fixation pathway (Bassham and Calvin, 1957), whereas Maize is distinguished by its use of the Hatch-Slack or C_4 pathway (Edwards, Kanai and Black, 1971) which gives the plant a much higher photosynthetic efficiency.

The tissues of the two types of cereal have fine structural differences corresponding to the physiological differences (Laetsch, 1969). The mesophyll cells of Calvin-type cereals, e.g. wheat and rye have monomorphic chloroplasts (Figs. A.2.1 and A.2.2). Two types of chloroplast are present in the Maize tissues. The bundle sheath cells have large closely packed chloroplasts which are virtually grana free. (Figs. A.2.3 and A.2.4). They are primarily concerned with starch production and under appropriate conditions will store considerable amounts of starch. (Fig. A.2.5).

Starch precursors are synthesized in the mesophyll cells which surround the bundle sheath. The mesophyll chloroplasts are similar in structure to those of the Calvin type, but have more regularly ordered grana and a higher peripheral reticulum content. (Fig. A.2.6).

The differences in chloroplast morphology would provide excellent

marker systems for distinguishing mixed aggregates on hybrids of the two cereal types, thus facilitating induced fusion studies. Spontaneous fusion occurring between bundle sheath and mesophyll protoplasts of Maize would be of physiological interest also. The product would again be readily identified.

Using the cereal protoplast isolation procedure of Evans, Keats and Cocking (1972) protoplasts were isolated in limited amounts from Wheat, Rye and Maize leaves. The latter was however particularly resistant leaving strands of bundle sheath tissue completely undigested. Enzyme concentrations were increased without success. An electron microscopic study of the protoplasts isolated demonstrated that the original chloroplast characteristics are retained in the isolated protoplast and confirmed the homogeneity of the Maize mesophyll protoplast isolate.

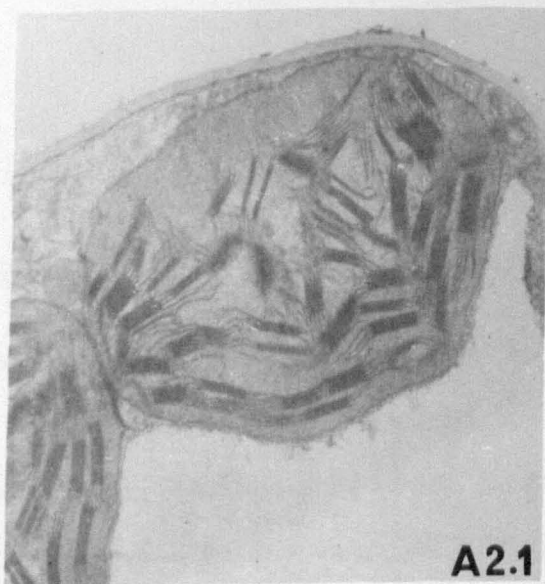
In the Maize leaf the bundle sheath and mesophyll cells are separated by a thick wall possibly containing lipids (Laetch 1969) and suberin. (O'Brien and Carr, 1970). (Fig. A.2.4 arrow). Electron microscopic examination of the tissues resistant to enzyme digestion shows that a thick dark staining layer remains surrounding the bundle sheath. (Fig. A.2.7). This explains the failure of the bundle sheath and mesophyll protoplasts to spontaneously fuse despite their rich plasmodesmatal supply. (Fig. A.2.4).

Using the technique of Edwards and Black (1971) bundle sheath cells were isolated by grinding maize leaf tissue in a mortar and pestle in the presence of a buffered salt solution. The isolated cells were then treated with wall digesting enzymes, a small yield of protoplasts being produced.

Thus cereal protoplasts with three distinguishable chloroplast forms can be isolated with varying degrees of efficiency.

Protoplasts from cereals

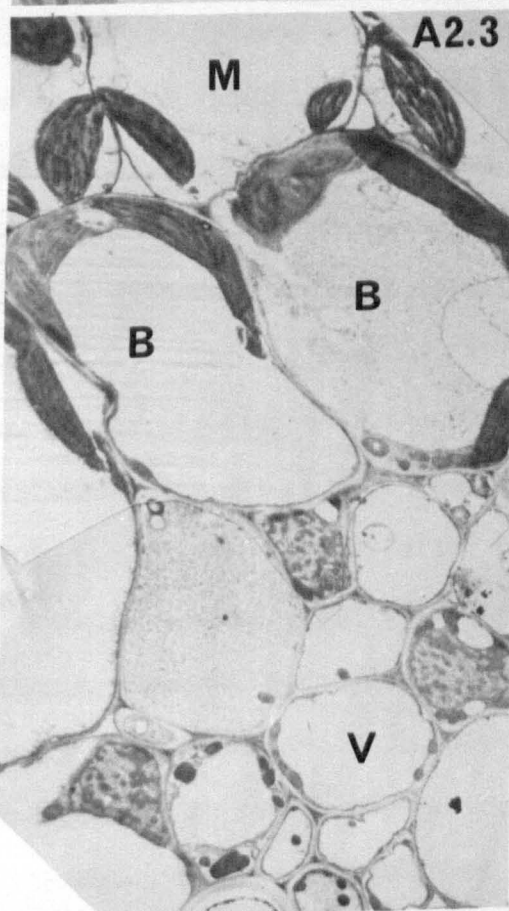
- | | | |
|-------------|--|----------|
| Figure A2.1 | A chloroplast in a wheat leaf. | x 20,000 |
| Figure A2.2 | A chloroplast in a rye leaf. | x 25,000 |
| Figure A2.3 | Maize leaf: Bundle sheath cells are located around the vascular tissue (V). Mesophyll cells lie outside the bundle sheath.(B=bundle sheath,M=mesophyll.) | x 4,500 |
| Figure A2.4 | Many plasmodesmata can be seen linking the bundle sheath and mesophyll cells. The chloroplasts in the former have very few if any grana and no peripheral reticulum,in this condition.(cf Figure A2.5) A dark staining layer in the wall is arrowed. | x 17,500 |
| Figure A2.5 | A bundle sheath chloroplast storing starch (s). The peripheral reticulum is now evident. | x 17,500 |
| Figure A2.6 | A mesophyll cell chloroplast.Many grana are present. The peripheral reticulum is arrowed. | x 30,000 |
| Figure A2.7 | Remnants of the enzymically digested wall.The resistant dark staining layer is arrowed. | x 15,000 |



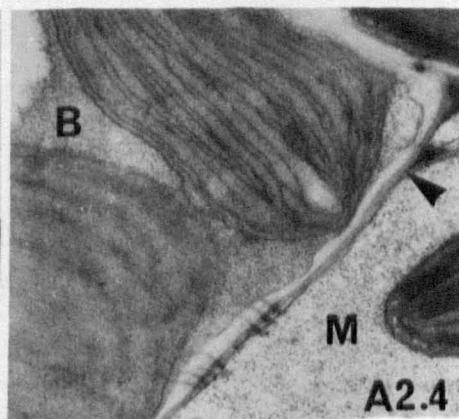
A2.1



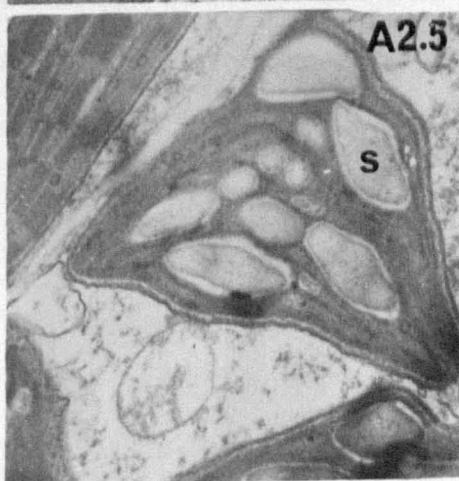
A2.2



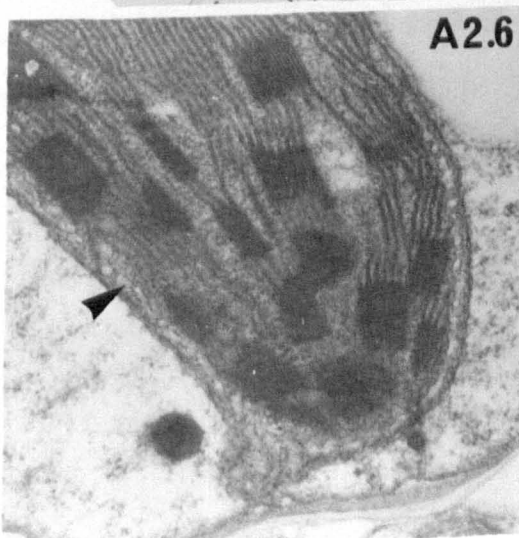
A2.3



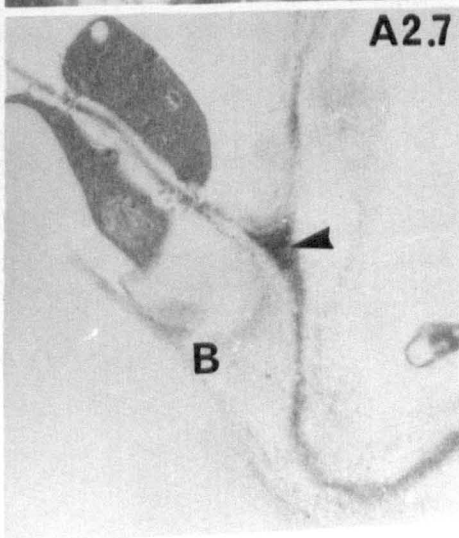
A2.4



A2.5



A2.6



A2.7

APPENDIX 2.2

Tobacco leaf epidermal protoplasts

Protoplasts have been isolated from both the upper and lower epidermes of the tobacco leaf (Sections 3.3.2 and 4.2.2). They form a highly homogeneous fraction. Stomatal guard cells remain totally undigested, (Fig. A.2.8) and very few if any vascular tissue cells are thought to yield protoplasts (c.f. Vasil & Vasil 1973). The latter have a very low degree of vacuolation in the tissue and the organellar content differs greatly from the isolated protoplasts.

During plasmolysis many vesicles form in the cytoplasm. (Fig. A.2.9) For this reason epidermal protoplasts might be useful material for uptake studies (see 3.3.3; Davey & Cocking, 1972). The cytoplasm contains few small chloroplasts, located near the nucleus (Fig. A.2.9). The grana are poorly developed. The mitochondria initially show plasmolysis effects (Prat, 1972), but recover shortly after isolation. The otherwise sparse cytoplasm contains several golgi like structures. (Fig. A.2.10).

In culture epidermal protoplasts divide, (Fig. A.2.11), form colonies and eventually regenerate into whole plants. (Davey, Frearson, Withers and Power, 1973). They regenerate more rapidly than protoplasts from mesophyll tissues. This is possibly due to the originally less differentiated condition of the chloroplasts. These would more readily undergo the changes necessary to the initiation of "greening" in the cultures, an essential stage in plant regeneration. (Sjollund and Wierer, 1971).

Tobacco leaf epidermal protoplasts

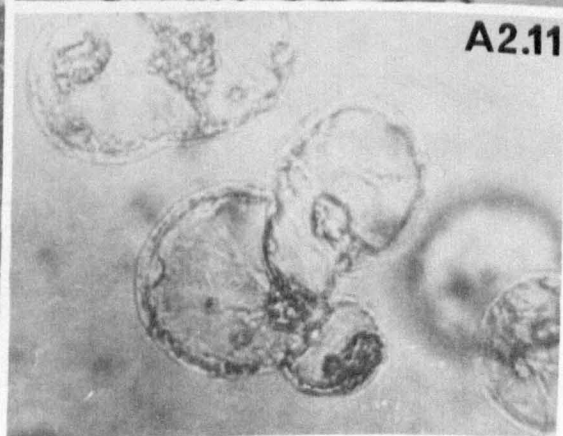
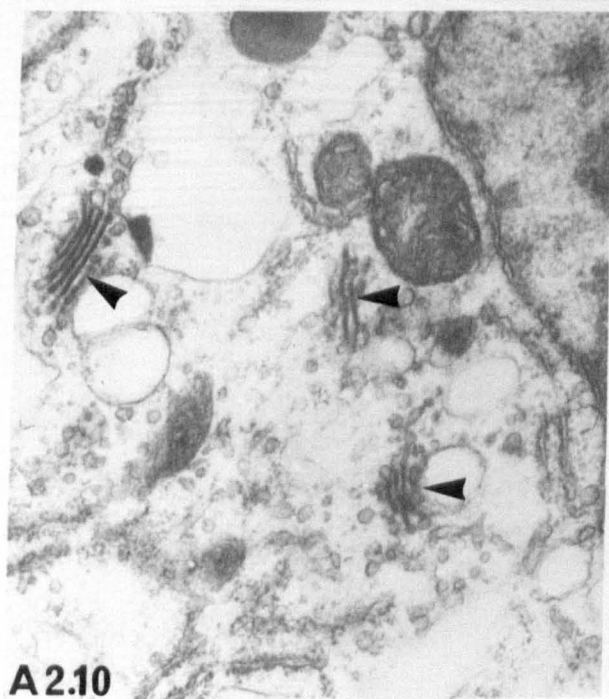
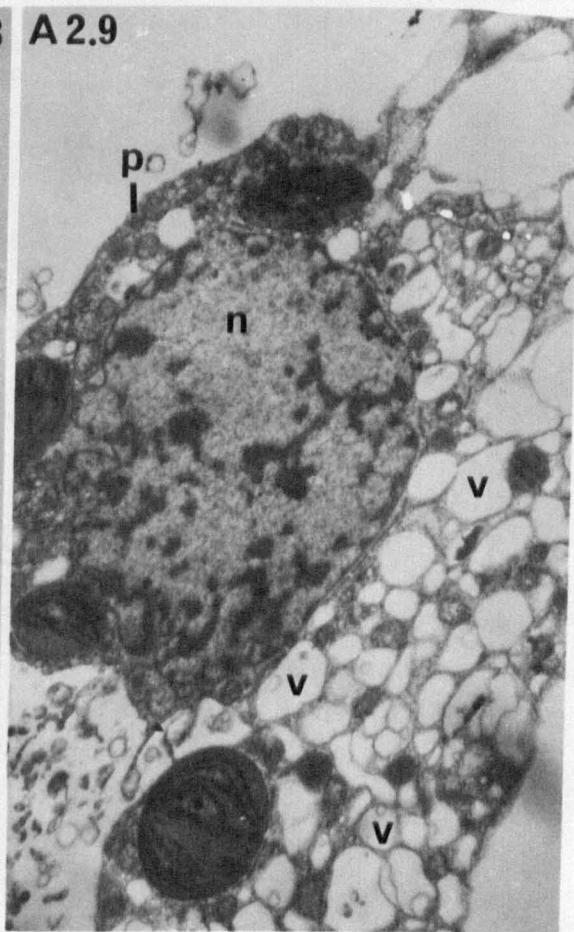
Figure A2.8 Stomatal guard cells. The adjacent epidermal protoplasts have digested away. x 5,000

Figure A2.9 Detail of an epidermal protoplast(Freshly isolated.)
Four small round chloroplasts are located near the nucleus (n). The cytoplasm contains many small vesicles (v). (p=plasmlemma) x 12,000

Figure A2.10 Golgi like structures (arrowed) commonly found in epidermal protoplasts. x 30,000

Figure A 2.11 An epidermal protoplast dividing in solid culture.
(Cultured for 8 days in TMS based medium.

x 225



APPENDIX 31. Calculation of standard deviation

$$s = \sqrt{v}$$

$$v = \frac{\sum (x - \bar{x})^2}{n - 1}$$

where s = standard deviation
 v = variance
 x = variable
 \bar{x} = mean
 n = number of observations of x

2. Comparison of means - using distribution of t

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s_d}$$

$$s_d = \sqrt{\frac{v_1}{n_1} + \frac{v_2}{n_2}}$$

where \bar{x}_1, \bar{x}_2 = means of samples 1 & 2
 s_d = standard deviation of $\bar{x}_1 - \bar{x}_2$
 v_1, v_2 = variances of samples 1 & 2
 n_1, n_2 = number of observations in samples 1 & 2

Using values of the distribution of t , values of p are determined according to the total degrees of freedom, d .

where p = probability

$$d = (n_1 - 1) + (n_2 - 1)$$

Data Set 1.

Field Number	Number of nuclei per protoplast a, absolute; b, % age, c, running mean % age								
	1			2			3		
	a	b	c	a	b	c	a	b	c
1	4	50	50	3	38	38	0	0	0
2	12	75	63	3	20	28	0	0	0
3	14	73	67	4	21	26	0	0	0
4	6	68	66	1	10	22	1	20	2.5
5	22	65	66	8	23	22	2	6	3
6	26	70	67	10	25	23	2	5	3.5
7	7	75	68	1	12	23	0	0	3
8	28	75	69	6	16	22	4	9	4
9	13	67	69	4	22	22	2	11	4.5
10	50	72	69	14	23	22	2	3	4.5
11	23	80	70	5	16	21	1	4	4.5
12	6	50	68	4	32	22	2	17	5.5
13	29	70	69	9	20	22	4	10	6
14	16	72	69	4	18	21	1	5	6

Data Set 2.

Method of assessment	Prepn. Number	Number of nuclei per protoplast								Total	percentage of multinucleates
		1	2	3	4	5	6	7	8		
Nuclear staining	1	234	43	10	9	0	0	0	0	300	20.5
Direct observation	1	170	25	4	0	0	0	0	1	200	15.0
Nuclear staining	2	94	38	6	10	0	2	0	0	150	38.0
Direct observation	2	135	38	15	2	4	4	2	2	200	32.5

Data Set 3.

Prepn. number	total axial length* (arbitrary units)	Mean protoplast diameter	Variance	Standard deviation (approx.)	percentage of multinucleates **
1	757	6.3	4.63	2.2	30.6
2	866	7.2	16.0	4.0	40.0
3	592	4.9	1.42	1.2	18.5

*2 axes of 60 protoplasts, total 120 axes.

**assessed by nuclear staining

1 : 2	t = 2.16	p = 3	(118 degrees of freedom)
2 : 3	t = 6.52	p = < 0.1	(118 degrees of freedom)
1 : 3	t = 6.16	p = < 0.1	(118 degrees of freedom)

Data Set 4 - 1 to 4, number of nuclei; 5, significance tests.

1.	186	46	95	178	83	117	Mean = 129
	232	42	134	160	155	121	Variance = 1917
	231	92	130	181	144	116	Standard deviation = 44
	72	97	109	113	148	174	
	75	97	180	110	83	166	
	86	144	182	119	122	119	
	146	171	189	155	121	127	
	129	146	188	173	140	135	
	135	103	192	75	128	81	
	38	96	201	75	134	120	
2.	320	204	265	577	611	478	Mean = 368
	318	327	301	504	608	341	Variance = 10430
	323	362	290	513	286	324	Standard deviation = 102
	375	353	293	344	275	321	
	365	351	381	335	281	524	
	363	239	387	342	314	539	
	300	244	397	288	311	538	
	292	229	442	276	330	526	
	305	259	419	290	328	498	
	298	263	437	613	481	345	
3.	658	349	1003	785	566	642	Mean = 708
	669	805	969	776	1044	651	Variance = 42221
	675	907	982	746	1041	628	Standard deviation = 205
	571	892	779	743	942	478	
	574	522	774	663	959	481	
	577	528	773	670	921	501	
	701	515	804	619	941	1516	
	713	805	816	496	707	870	
	380	814	820	552	716	501	
	360	932	810	555	692	481	
4.	920	748	1189	831	1287	1466	Mean = 1289
	760	881	996	899	1917	1216	Variance = 173259
	860	720	1068	938	1947	1222	Standard deviation = 416
	803	1550	1175	1817	1873	1196	
	791	1560	1226	1733	1907	1680	
	788	1575	1131	1838	1102	1462	
	753	1545	1141	1769	1092	2258	
	777	1152	1130	1807	1134	2232	
	769	1211	987	1309	1441	1705	
	864	1010	1175	1290	1442	1692	
5.	1 : 2	t = 16.8	p = <	0.001	(118 degrees of freedom)		
	2 : 3	t = 11.5	p = <	0.001	"	"	"
	3 : 4	t = 9.9	p = <	0.001	"	"	"

Data Set 5 - 1, Consistency test, 2, Background test, 3, Mask Size Test.

1.	407	395	403
	405	386	412
	402	387	438
	407	381	418
	389	407	401
	408	405	407
	404	389	407
	410	380	400
	409	390	405
	409	407	401

Mean = 402

Variance = 137

Standard deviation \approx 12

2.	a.	100	70	45
	b.	1750	1706	1675
	c.	1750	1436	640

a = relative background illumination

b = uncorrected readings

c = corrected readings

3.	270	377	489	799
	259	329	566	638
	235	403	564	670
	205	331	555	757
	275	404	564	781
	234	342	495	792
	215	396	500	838
	223	328	544	684
	200	419	473	684
	224	394	547	668

Means:	236	372	530	730
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Data Set 6.

Pretreatment	Number of nuclei per protoplast						Total	percentage of multinucleates
	1	2	3	4	5	6		
Control	119	22	7	1	0	1	150	20.6
Stripped leaf pieces placed in 3% sorbitol for 2h	132	13	4	0	1	0	150	12.0
Stripped leaf pieces placed in distilled water for 2h	140	8	2	0	0	0	150	6.6
Whole leaf placed in distilled water for 2h	141	7	1	1	0	0	150	7.3

Data Set 8.

Enzyme incubation medium (Plus 13% w/v sorbitol)			Number of uni- nucleates	Number of multi- nucleates	Total	% age of multi- nucleates
Meicelase P		Macerozyme				
Crude	Purified					
2		0.4	110	35	145	24.2
	0.2	0.4	103	43	145	29.7
4		0.4	116	14	130	10.7
	0.4	0.4	111	19	130	14.6
10		0.4	123	22	145	15.2
	1.0	0.4	115	25	140	17.9

Data Set 9.

Age of Plant material, days	Temp. °C.	Number of uni- nucleates	Number of multi- nucleates	Total	% age of multi- nucleates
50	20	144	16	180	8.9
50	30	157	18	175	10.3
50	40	149	16	165	9.7
82	20	143	27	170	15.8
82	30	120	40	160	25.0
82	40	149	56	155	36.1

Data Set 10.

Enzyme incubation medium made up in	Prepn. No.	Number of uni-nucleates	Number of multi-nucleates	Total	% of multi-nucleates	Yield, $\times 10^6$ protoplasts per g.	
						Total	Yield of multi-nucleates
Distilled water	1	105	35	140	24.3	0.99	0.24
	2	122	28	150	18.7	0.85	0.16
TMS	1	96	34	130	26.1	1.30	0.34
	2	125	35	160	19.4	1.28	0.25

Data Set 11.

Age of Plant material days	Number of uni-nucleates	Number of multi-nucleates	Total	% age of multi-nucleates	Yield		
					Total		multi-nucleates relative %
					absolute $\times 10^6$ per g	relative %	
44	109	46	150	30.6	0.43	31	9.5
51	90	60	150	40.0	0.64	45	18.0
58	95	70	165	42.5	0.95	68	28.8
68	102	58	160	36.2	1.31	93	33.7
75	108	52	160	32.5	1.40	100	32.5
82	86	54	140	38.5	1.22	87	33.5

Data Set 12.

Age of culture days	Culture number	Number of uni-nucleates	Number of multinucleates	Total	% of multinucleates
0	1	124	36	160	22.5
0	2	133	37	170	21.8
12	1	148	12	160	7.5
15	2	113	12	125	9.6
20	1	114	4	118	3.4
23	2	175	5	180	2.8

Data Set 13.Control Culture

157	441	467	462	289	497
129	462	477	612	211	406
356	96	574	178	173	781
332	72	321	533	354	198
552	440	838	657	169	783
841	427	162	422	343	197
488	308	558	644	375	614
658	80	429	418	670	759
953	407	66	545	366	602
350	530	50	668	505	710
330	688	291	501	882	616
375	328	522	1054	415	205
60	781	341	500	417	197
707	361	479	1011	422	311
645	367	399	532	580	185
562	461	308	334	559	563
481	948	642	529	890	603
270	454	124	329	581	587
432	660	711	299	502	899
561	158	253	305	881	904

Mean = 457

Data Set 14 - 1 and 3, nuclei in "single" cells; 2 and 4, nuclei in "clumped" cells.

1.	203	119	174	687	217	63
	433	27	928	86	339	284
	392	122	88	406	321	96
	287	1053	196	297	135	117
	114	149	313	647	800	166
	329	354	290	344	124	356
	316	371	385	275	181	113
	235	216	127	436	830	66
	475	308	463	412	402	71
	51	473	268	240	130	158

Mean = 289

2.	796	1714	2799	1370	1057	1175
	1422	86	165	1448	196	564
	337	2261	150	831	766	637
	1918	1051	670	1047	547	561
	2124	2237	1186	507	553	139
	837	2038	600	1010	121	1257
	2983	317	432	1517	272	554
	2363	129	846	716	1096	2736
	1144	1289	557	2266	655	686
	786	260	827	508	933	798

Mean = 1014

3.	441	107	292	101	1278	559
	239	134	931	138	125	577
	142	74	582	585	138	792
	687	39	84	136	260	288
	441	129	268	75	687	102
	581	257	212	458	252	301
	108	443	31	85	208	475
	429	595	1271	133	448	926
	198	882	149	35	385	325
	132	272	139	246	37	144

Mean = 343

4.	2661	723	650	657	2582	668
	3244	710	1248	1180	1611	882
	3740	743	838	849	722	765
	873	666	1491	638	2161	319
	315	764	1609	2380	717	1355
	341	1357	2068	616	3245	1510
	852	752	1334	719	1339	599
	2381	659	74	901	744	422
	724	656	1420	1490	77	525
	1482	1176	788	1499	3810	905

Mean = 1146